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# Imidazo[1,2-*a*]pyridine derivatives as inhibitors of TNF-α expression in T cells

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**Abstract**—Novel hexahydroimidazo[1,2-*a*]pyridines prepared by the addition of ethyl (1-benzylimidazolidin-2-ylidene)acetate (**2**) to the fungal metabolite podoscyphic acid (**1a**) and esters of **1a** have been evaluated for their ability to inhibit the inducible TNF-α promoter activity in T cells. The methyl ester **3b** is the most potent, inhibiting the TNF-α driven reporter gene expression in Jurkat T cells with an $IC_{50}$-value of 2.0 μg/ml (3.6 μM). In addition, compound **3b** inhibited the inducible TNF-α production in the myelomonocytic U937 cells with an $IC_{50}$-value of 4.6 μM.


## 1. Introduction

The human TNF-α gene is expressed in a variety of cell types (e.g., T and B lymphocytes and macrophages). It is one of the major proinflammatory cytokines which regulates further cytokine induction, especially of IL-1β and IL-6, and plays a role in many human diseases including cancer and immune disorders, as well as in inflammatory diseases like septic shock, rheumatoid arthritis and Crohn's disease.[1] In quiescent cells the biosynthesis of TNF-α is tightly controlled. However, diverse extra-cellular stimuli, including antigens, viruses, bacterial products, cytokines or exposure to calcium ionophores, lead to a rapid induction of TNF-α gene expression. The regulation of TNF-α gene expression in eukaryotic cells is complex with differences across species and cell types. The common end point of these diverse signal transduction pathways is the activation of transcription factors of the NF-AT, NF-κB and ATF2/Jun family, which bind to the corresponding binding sites in the promoter of the TNF-α gene and induce transcription. It has recently been shown that distinct transcription factor complexes form on different activator recognition sites on the TNF-α promoter in response to distinct extra-cellular stimuli.[2] Drugs which block TNF-α in clinical practice or in clinical trials are either monoclonal antibodies to TNF-α or interfere with TNF-α receptors, examples are Enbrel (Amgen/Wyeth), Remicade (Centocor/Schering-Plough/Tanabe Sieyaku) and Humira (Abbott).[3,4] An example is the teratogenic drug thalidomide which was shown to have both anti-inflammatory and anti-oncogenic properties.[5] Novel low molecular weight inhibitors, which specifically interfere with components of the different intracellular signalling pathways or inhibit the activation of the transcription factors responsible for the expression of TNF-α, could be useful as novel therapeutics in inflammation.[6,7] During an evaluation of the possibility to create a diverse library of semisynthetic derivatives based on the strongly electrophilic fungal metabolite podoscyphic acid (**1a**), imidazo[1,2-*a*]pyridine derivatives were shown to be obtained by the addition of imidazolidin-2-ylideneacetic acid derivatives with **1**. When screened for various biological activities, the products were found to be efficient inhibitors of the inducible human TNF-α promoter activity as well as the inducible synthesis of TNF-α.

## 2. Preparation of imidazo[1,2-*a*]pyridine derivatives

Podoscyphic acid (**1a**) is produced by the fungus *Podoscypha petalodes* and was originally isolated as an inhibitor of retroviral reverse transcriptases.[8] It is a

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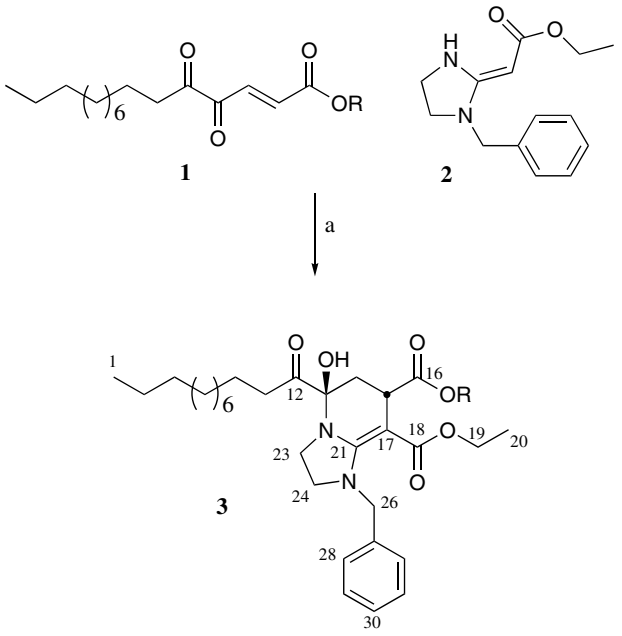


**Figure 1.** (a) R=H; (b) R=Me; (c) R=Et. Reaction and conditions: (a) triethylamine, $CH_2Cl_2$, reflux 3 h (yield 52–83%).

highly reactive compound, with a combination of electrophilic functionalities that enables it to take part in one or several nucleophilic additions. An interesting nucleophilic partner is the enaminoester (**2**) possessing a highly polarised double bond.[9] It has previously been utilised for the synthesis of tetrahydropyridines and piperidines through cyclization reactions with a variety of electrophiles and 1,3-dipoles.[10] Presumably, the enamine attacks the activated carbon–carbon double bond on C-2 whereafter the secondary amine nitrogen adds to the C-4 keto group, yielding the novel imidazo[1,2-*a*]pyridine **3a**. The reaction could also be performed with esters of podoscyphic acid, and the methyl ester **3b** as well as the ethyl ester **3c** were also prepared. The relative configuration was suggested by NOEs observed between the 13-OH and 15-H as well as 14-Hβ, and between 11-$H_2$ and 14-Hα (Figure 1).

## 3. Effect on hTNF-α promoter activity

The assay used in this investigation is based on the expression of a human TNF-α transcriptional reporter in Jurkat T cells. Transfection of Jurkat cells with a hTNF-α promoter driven luciferase reporter gene plasmid and stimulation with 32 nM TPA and 2 μM ionomycin resulted in a 17- to 20-fold activation over the basal level of luciferase expression. The effects of the imidazo[1,2-*a*]pyridine derivatives are shown in Figure 2. The methyl ester **3b** is the most potent, inhibiting the TPA/ionomycin stimulated expression of the TNF-α promoter mediated luciferase expression in Jurkat cells with an $IC_{50}$-value of 2.0 μg/ml (3.6 μM). The free acid **3a** and the ethyl ester **3c** were less potent with $IC_{50}$-values of 5–10 μg/ml (9.5–19 μM). Treatment of the cells with the ethyl ester **3c** actually resulted in an incomplete inhibition of TNF-α promoter driven reporter gene expression, with a remaining activity of 25% of the induced control even at the highest concentration tested (55 μM). The parent compound podoscyphic acid (**1**) showed no inhibitory effects on the TPA/ionomycin induced TNF-α promoter activity in Jurkat cells.

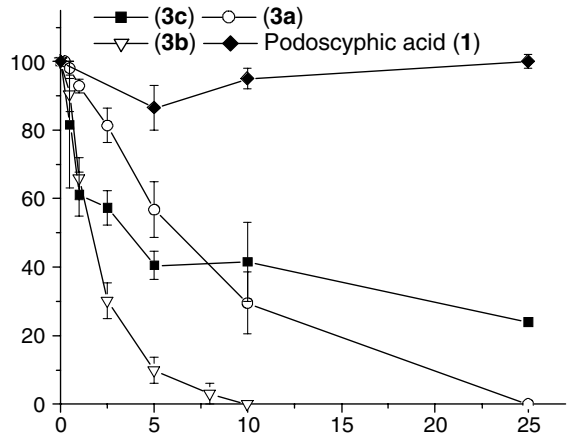


**Figure 2.** Effect of podoscyphic acid (**1a**) and the imidazo[1,2-*a*]pyridine derivatives **3a**, **3b** and **3c** on hTNF-α promoter activity. See Section 6 for details.

## 4. Effect on TNF-α production

The influence of the compounds on TNF-α production was investigated in myelomonocytic U937 leukaemia cells which have been shown to release significant amounts of TNF-α following TPA treatment.[11] Pretreatment of U937 cells with the test compounds and stimulation with 50 ng/ml TPA resulted in a dose dependent inhibition of TNF-α synthesis, which was comparable to the results obtained in the hTNF-α promoter reporter gene assay. As shown in Figure 3, the methyl ester **3b** blocked the TNF-α synthesis with an $IC_{50}$-value of 2.5 μg/ml (4.6 μM). Compound **2** was less active with

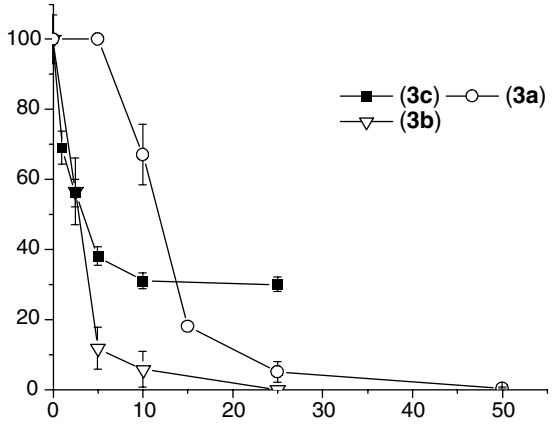


**Figure 3.** Effect of the imidazo[1,2-*a*]pyridine derivatives **3a**, **3b** and **3c** on TNF-α production. U937 cells were pretreated for 1 h with or without test compounds and stimulated with 50 ng/ml TPA for additional 16 h. TNF-α concentrations from cell supernatants were determined by ELISA. Control (100%): stimulation only (905 pg/ml TNF-α). Results are representative of three independent experiments.

**Table 1.** Effect of compound (**3**) on SEAP or luciferase reporter gene expression in HepG2, HeLa S3 and Jurkat cells

| Reporter gene assay | Cell line | Stimulus | (**3**) $IC_{50}$ μg/ml (μM) |
|---|---|---|---|
| 3 × AP-1–SEAP | HeLa S3 | TPA (25 ng/ml) | 2.5 (4.6) |
| 5 × GAS/ISRE–SEAP | HeLa S3 | IFN-α (0.5 ng/ml)/IFN-γ (10 ng/ml) | 2.5 (4.6) |
| 8 × IL-6RE II–SEAP | HepG2 | IL-6 (10 ng/ml) | 10 (18.4) |
| 5 × NF-AT/AP-1–SEAP | Jurkat | TPA (10 ng/ml)/ionomycin (2.5 μL) | 2.5 (4.6) |
| 5 × NF-κB–SEAP | Jurkat | TPA (10 ng/ml)/ionomycin (2.5 μL) | 2.5 (4.6) |
| HCOX-2–pro-Luc | Jurkat | TPA (10 ng/ml)/ionomycin (2.5 μL) | 2.5 (4.6) |

The cell lines were transiently transfected with the indicated reporter gene constructs and the expression of the reporter genes was induced as described in Section 6.

$IC_{50}$-values of 10–15 μg/ml, while **3c**, as in the case for the hTNF-α transcriptional reporter, only caused a partial inhibition of TNF-α synthesis.

In order to test the specificity of the most active compound, we determined the effect of **3b** on the GAS/ISRE, AP-1, NF-κB, NF-AT and human COX-2 promoter driven expression of the reporter genes SEAP or luciferase in HeLa S3 and Jurkat cells. Compound **3b** inhibited the AP-1, NF-κB, GAS/ISRE and NF-AT mediated SEAP expression in transiently transfected HeLa S3 or Jurkat cells with $IC_{50}$-values of 2.5 μg/ml (4.6 μM, see Table 1). The IL-6 induced expression of the IL-6RE driven reporter plasmid in transiently transfected HepG2 cells was inhibited by **3b** to a 4-fold lesser extent. Beside TNF-α, the inducible isoform of the cyclooxygenase COX-2 is upregulated in many cell types by proinflammatory stimuli and it has been shown that the signalling mechanisms governing COX-2 expression also contribute to the inducible TNF-expression.[12,13] We therefore investigated the influence of **3b** on a human COX-2 transcriptional reporter. The compound inhibited the TPA/ionomycin stimulation of the COX-2 promoter activity with an $IC_{50}$-value of 2.5 μg/ml (4.6 μM).

Cytotoxic properties of the compounds were evaluated against various cultured neoplastic cell lines. As shown in Table 2, cytotoxic activities against most tested cell lines could be observed for all compounds with $CC_{50}$-values starting from 10 to 25 μg/ml (18–20.7 μM) during a 48-h incubation period. The $CC_{50}$-values for all compounds against HepG2 cells were approximately 2- to 5-fold higher.

It has recently been shown that the expression of TNF-α in T cells is regulated by several distinct MAP kinase pathways.[14] Inhibition of ERK or p38 activating pathway by PD98059 or SB203580 resulted in a strong inhibition of TNF-α promoter dependent transcription as well as TNF-α production. In order to determine whether **3b** affects the activation of the ERK and p38 MAPK, Western blot analyses were performed with antibodies specific for the phosphorylated form of ERK or p38. As shown in Figure 4, treatment of Jurkat cells with TPA/ionomycin resulted in a strong induction of ERK1/2 phosphorylation. The p38 phosphorylation was induced to a lesser extent. Pretreatment of the cells with up to 10 μg/ml of **3b** showed no inhibition of ERK1/2 or p38 phosphorylation. The reduction of ERK1/2 and p38 phosphorylation at higher concentrations is probably due to cytotoxic effects.

**Table 2.** Cytotoxic activities of the synthesized compounds towards various tumour cell lines in vitro

| Cell line | **3a** $CC_{50/90}$ (μg/ml) | **3b** $CC_{50/90}$ (μg/ml) | **3c** $CC_{50/90}$ (μg/ml) |
|---|---|---|---|
| Colo 320 | 10/50 | 10/50 | 10–25/>50 |
| HeLa S3 | 25–50/– | 10–25/25 | 10/50 |
| HepG2 | >50/– | 50/– | 50/– |
| RAW 264.2 | 10–25/50 | 5–10/25 | 5–10/25 |
| HL-60 | 10–25/50 | 10–25/25 | 10–25/25 |
| Jurkat | 10–26/50 | 10–25/50 | 10–25/50 |
| U937 | 10–25/25 | 10–25/25 | 10–25/25 |

Both $CC_{50}$ and $CC_{90}$-values are given.

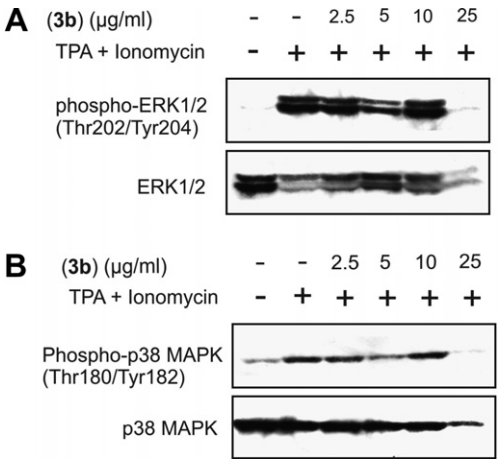


**Figure 4.** Effect of **3b** on ERK1/2 (A) and p38 (B) phosphorylation. Jurkat cells were pretreated for 1 h with or without test compounds and induced with 25 ng/ml TPA and 2.5 μM ionomycin for 30 min. Subsequently total cell extracts were prepared and equal amounts of protein analyzed by Western blotting for A: ERK1/2 phosphorylation with a phospho-ERK1/2 antibody and B: p38 phosphorylation with a phospho-p38 antibody.

These results indicate that the inhibition of TNF-α promoter activity by **3b** did not result from the interference with two signalling pathways responsible for the activation of the transcription factor ATF2, which cooperates functionally with NF-AT family proteins to activate TNF-α gene transcription in T cells.[15,16] The transcription factors of the NF-AT family are expressed in most immune-system cells and play a pivotal role in the

transcription of cytokine genes and other genes critical for the immune response. NF-AT is also notable for its ability to bind cooperatively with transcription factors of the AP-1 family to composite NF-AT:AP-1 sites, as found in the regulatory regions of many genes that are inducibly transcribed by the immune-system cells.[17] The reporter gene vector pGE3-NF-AT used in this work contains the distal NF-AT:AP-1 site of the human IL-2 promoter. It is therefore conceivable that **3b** interferes with the signalling cascade leading to NF-AT activation.[18] In addition, compound **3b** inhibited the NF-κB, AP-1 and STAT1 mediated gene expression but the exact cellular target remains to be determined. Interestingly **3b** did not inhibit the inducible expression of the COX-2 and iNOS proteins in LPS/IFN-γ stimulated murine monocytic RAW 264.7 cells (data not shown), which indicates a preferential target within the signalling pathways leading to TNF-α expression in T cells.

## 5. Conclusions

A set of novel imidazo[1,2-*a*]pyridine derivatives, which inhibit the inducible expression of TNF-α in T cells, have been prepared from podoscyphic acid. Since TNF-α is one of the major proinflammatory cytokines, the compounds may serve as important tools for increasing our understanding about the mechanisms by which it acts as well as for the development of drugs interfering with the signalling pathways leading to the inducible expression of the TNF-α gene. The methyl ester **3b** was found to be more potent than the free acid **3a** and the ethyl ester **3c**, but a more comprehensive study is needed to establish clear structure–activity relationships. Further investigations on the mode of action of the compounds to characterize the cellular targets are now under way.

## 6. Experimental

### 6.1. Chemistry, general procedures

Unless otherwise noted, chemicals were of p.a. quality and obtained from commercial suppliers and used without further purification. TLC analyses were made on 'Merck DC-Alufolien Kieselgel 60 F254' $SiO_2$ plates, visualised by spraying with anisaldehyde/sulfuric acid and warming to 120 °C. The MS spectrum (direct inlet, 70 eV) was recorded with a JEOL SX102 spectrometer, and NMR spectra (in $CDCl_3$) with a Bruker ARX 500 spectrometer at 500 MHz ($^1H$) and 125 MHz ($^{13}C$). The chemical shifts are reported in ppm with the solvent signals ($\delta_H = 7.26$ and $\delta_C = 77.0$) as reference. COSY, HMQC, HMBC and HETCOR experiments were recorded with gradient enhancements using sine shaped gradient pulses. Flash chromatography employed Grace Amicon silica gel 60 (35–70 μm).

Podoscyphic acid (**1a**) was synthesised,[19] the methyl ester **1b** was prepared with diazomethane in diethyl ether, while the ethyl ester **1c** was synthesised.[19] Ethyl (1-benzylimidazolidin-2-ylidene)acetate (**2**) was prepared from *N*-benzyl-1,2-diaminoethane and the imidate obtained from ethyl cyanoacetate.[20]

The structures of the imidazo[1,2-*a*]pyridine derivatives were determined by high resolution NMR spectroscopy and mass spectrometry. The $^{13}C$ NMR signal for C-17 is broadened and weak in all derivatives, also in compound **2**, and was actually not observed in the $^{13}C$ NMR spectrum of the free acid **3a**. However, high resolution MS experiments clearly show that the composition of **3a** is $C_{30}H_{44}N_2O_6$.

**6.1.1. 1-Benzyl-5-dodecanoyl-5-hydroxy-1,2,3,5,6,7-hexahydro-imidazo[1,2-*a*]pyridine-7,8-dicarboxylic acid-8-ethyl ester (3a).** To a stirred solution of podoscyphic acid (**1a**) (50 mg, 0.177 mmol) and **2** (43.5 mg, 0.177 mmol) in $CH_2Cl_2$ (5 ml) was added a catalytic amount of triethylamine (5 μl) and the reaction mixture was refluxed and monitored on TLC until no more starting material could be detected (approx. 3 h). The solvent was removed in vacuo and the residue was purified by chromatography on silica gel, eluted with $CH_2Cl_2$/MeOH 30:1.77 mg pure product (83%) was obtained as a yellowish waxy solid; $^1H$ NMR (500 MHz, $CDCl_3$): $\delta$ 0.84 (t, $J = 7.0$ Hz, 3H, H-1), 1.24 (t, $J = 7.0$ Hz, 3H, H-20), 1.20–1.28 (m, 16H, H2–H9), 1.54 (m, 2H, H-10), 1.83 (dd, $J = 14.7$ and 4.1 Hz, 1H, H-14), 2.62 (m, 1H, H-11), 2.83 (m, 2H, H-11), 3.54 (t, $J = 3.0$ Hz, 1H, H-15), 3.62 (m, 1H, H-24), 3.66 (m, 2H, H-23), 3.99 (m, 1H, H-24), 4.21 (m, 2H, H-19), 4.51 (d, $J = 15$ Hz, 1H, H-26), 4.76 (d, $J = 15$ Hz, 1H, H-26), 4.96 (br s, 1H, H–(-OH)–13), 7.29–7.40 (m, 5H, H-28–H-30); $^{13}C$ NMR (125 MHz, $CDCl_3$): $\delta$ 13.9 (C-20), 14.0 (C-1), 22.5 (C-2), 23.0 (C-3), 29.0 (C-4), 29.2 (C-5), 29.3 (C-6), 29.4 (C-7), 29.5 (C-8), 29.5 (C-9), 31.5 (C-14), 31.8 (C-10), 37.2 (C-11), 41.2 (C-15), 43.1 (C-24), 47.1 (C-23), 50.9 (C-26), 63.1 (C-19), 88.8 (C-13), 128.5 (C-30), 129.2 (C-28), 129.3 (C-29), 131.4 (C-27), 162.5 (C-21), 166.0 (C-18), 174.2 (C-16), 207.2 (C-12); HRFABMS: 529.3296 ($M+H^+$, $C_{30}H_{45}N_2O_6$ requires 529.3278).

**6.1.2. 1-Benzyl-5-dodecanoyl-5-hydroxy-1,2,3,5,6,7-hexahydro-imidazo[1,2-*a*]pyridine-7,8-dicarboxylic acid-8-ethyl ester-7-methyl ester (3b).** The methyl ester of podoscyphic acid (**1b**) (46 mg, 0.155 mmol) and 1.2 equiv of **2** (46 mg, 0.186 mmol) were reacted as described for compound **3a**, to afford 44 mg (52%), as a yellowish waxy solid; $^1H$ NMR (500 MHz, $CDCl_3$): $\delta$ 0.87 (t, $J = 7.0$ Hz, 3H, H-1), 1.14 (t, $J = 7.1$ Hz, 3H, H-20), 1.18–1.29 (m, 16H, H-2–H-9), 1.54 (m, 2H, H-10), 2.16 (dd, $J = 13.8$ and 6.9 Hz, 2H, H-14), 2.67 (d, $J = 13.2$ Hz, 1H), 2.65 (m, 2H, H-11), 2.96 (t, $J = 8.2$ Hz, 1H, H-23), 3.23 (m, 1H, H-24), 3.35 (m, 2H, H-23, H-24), 3.71 (s, 3H, H–$R_2$=Me ), 4.0 (d, $J = 5.5$ Hz, 1H, H-15), 4.05 (m, 2H, H-19), 4.45 (d, $J = 15.2$ Hz, 1H, H-26), 4.91 (d, $J = 15.2$ Hz, 1H, H-26), 4.96 (br s, 1H, H–(-OH)–13), 7.15–7.35 (m, 5H, H-28–H-30); $^{13}C$ NMR (125 MHz, $CDCl_3$): $\delta$ 14.0 (C-1), 14.5 (C-20), 22.6 (C-2), 23.4 (C-3), 29.0 (C-4), 29.1 (C-5), 29.2 (C-6), 29.2 (C-7), 29.3 (C-8), 29.3 (C-9), 31.8 (C-10), 34.5 (C-14), 36.8 (C-11), 37.8 (C-15), 42.2 (C-23), 47.3 (C-24), 52.2 (C–$R_2$=Me), 55.4 (C-26), 58.8 (C- 19), 72.2 (C-17), 85.6 (C-13), 125.2 (C-30), 127.2

(C-28), 128.6 (C-29), 137.8 (C-27), 159.7 (C-21), 166.2 (C-18), 177.7 (C-16), 208.9 (C-12); HRFABMS: 543.3444 ($M+H^+$, $C_{31}H_{47}N_2O_6$ requires 543.3435).

Compound **3b** was also prepared by treating **3a** (60 mg, 0.113 mmol) in diethyl ether with diazomethane at room temperature. Forty milligrams (63%), identical with **3b** prepared above, was obtained.

**6.1.3. 1-Benzyl-5-dodecanoyl-5-hydroxy-1,2,3,5,6,7-hexahydro-imidazo[1,2-*a*]pyridine-7,8-dicarboxylic acid diethyl ester (3c).** The ethyl ester of podoscyphic acid (**1c**) (46 mg, 0.148 mmol) and 1.2 equiv of **2** were reacted as described for compound (**2**) to afford 46 mg (56%) **3c** as a yellowish waxy solid. $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 0.89 (t, $J$ = 6.9 Hz, 3H), 1.17 (t, $J$ = 7.0 Hz, 3H), 1.17–1.35 (m, 19 H), 1.60 (m, 2H), 2.25 (m, 1H), 2.67 (m, 2H), 3.0 (t, $J$ = 5.5 Hz, 1H), 3.26 (m, 1H), 3.35 (m, 1H), 3.99 (d, $J$ = 5.3 Hz, 1H), 4.07 (m, 2H), 4.18 (m, 2H), 4.43 (d, $J$ = 15.3 Hz, 1H), 4.94 (d, $J$ = 15.3 Hz, 1H), 5.57 (br s, 1H), 7.15–7.35 (m, 5H); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 14.0, 14.1, 14.5, 22.6, 23.5, 29.1, 29.2, 29.3, 29.4, 29.5, 31.8, 34.3, 36.9, 37.9, 42.2, 47.2, 48.3, 55.4, 58.7, 61.1, 72.2, 85.6, 127.4, 128.3, 128.6, 137.8, 159.7, 166.2, 177.4, 208.9; HRFABMS: 557.3569 ($M+H^+$, $C_{32}H_{49}N_2O_6$ requires 557.3591).

### 6.2. Biological assays

Jurkat cells (ATCC TIB 152), U937 cells (ATCC CRL-1593), HL-60 cells (ATCC CCL 240), THP-1 cells (DSMZ ACC 16) and RAW 264.7 cells (ATCC TIB-71) were grown in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) and 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. HepG2 cells (ATCC HB 8065), COLO 320 cells (DSMZ ACC 144) and HeLa S3 (ATCC CCL 2.2) were maintained in DMEM supplemented with 10% foetal calf serum. The assays for cytotoxicity were carried out as described previously.[21]

**6.2.1. Reporter gene assays.** The 1.2 kb human TNF-α promoter was amplified by PCR from genomic DNA extracted from HeLa S3 cells as described recently.[22] The PCR product was cloned into the XhoI-HindIII site of the pGL3-Basic vector (Promega) to generate the TNF-α promoter driven luciferase reporter plasmid pJR-TNF-pro. The reporter plasmid pMW-IRF7 was constructed by cloning eight copies of a class II IL-6 responsive element (IL-6RE II) of the IRF promoter immediately upstream of the thymidine kinase promoter driven SEAP reporter gene. The plasmid pRL-CMV for normalizing transfection efficiency was obtained from PROMEGA (Dual-Luciferase-Reporter-Assay). The reporter plasmids pGE3-NF, pTK-AP-1, pGE3-GAS/ISRE and pGE3-NF-AT have been described recently.[23] Transfection of HepG2 cells was performed by electroporation (BioRad, GenePulser) as described.[23] The activity of the SEAP in the culture medium was determined 24 h after transfection using the Phospha-Light chemiluminescent assay (TROPIX, MA) according to the manufacturer's instructions with a luminometer. Jurkat cells were electroporated ($6 \times 10^7$ cells/ml in 0.2 ml 0.5× HEBS buffer, 50 μg of the pJR-TNF-pro vector or of the pGE3-NF-AT vector) and seeded in 96-well plates ($6 \times 10^5$ cells/ml in OPTIMEM containing 10% FCS) with and without test compounds. Reporter gene expression was induced with 32 nM TPA and 2 μM ionomycin. The reporter gene activity was measured 24 h after transfection using the luciferase assay system (Promega) according to the manufacturer's instructions with a luminometer in case of the pJR-TNF-pro vector or as described above in case of the pGE3-NF-AT vector. Transfection of HeLa S3 cells with pGE3-NF1, pGE3-GAS/ISRE or pTK-AP-1 and determination of the activity of the expressed SEAP was performed as described previously.[24]

**6.2.2. ELISA.** TNF-α production in U937 cells pretreated for 1 h with or without test compounds and stimulated with 50 ng/ml TPA for additional 16 h was determined by ELISA (QuantiGlo™ human TNF-α ELISA, R&D Systems, UK) according to the manufacturer's instructions.

**6.2.3. Western blots.** Jurkat cells were starved for 24 h in RPMI 1640 medium containing 0.5% FCS, treated for 1 h with test compounds and induced with 25 ng/ml TPA and 2.5 μM ionomycin for 30 min. Total cell extracts were prepared using RIPA detergent buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, 1.5 mM $KH_2PO_4$, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1 μM $Na_3VO_4$, complete protease inhibitor cocktail 1:50 (Roche Diagnostics, Germany)) and cell extracts (50–100 μg protein) were subjected to 10% SDS–PAGE, transferred onto a nitrocellulose membrane and probed with antibodies specific for the phosphorylated forms of ERK1/2 and p38 (New England Biolabs, Frankfurt), and then with the appropriate secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were visualised by the enhanced chemiluminescent detection system (ECL system, Amersham International, UK). After stripping of the membrane, the blots were reprobed with anti-ERK1/2 or anti-p38 antibodies and developed as described above.

## Acknowledgment

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