Cycloepoxydon, 1-Hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene and 1-Hydroxy-2-hydroxymethyl-3-pent-1,3-dienylbenzene, New Inhibitors of Eukaryotic Signal Transduction

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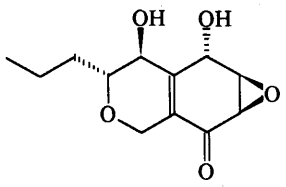
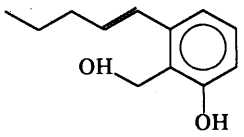
In a screening for new inhibitors of NF-κB and AP-1 mediated signal transduction pathways in COS-7 cells using secreted alkaline phosphatase (SEAP) as a reporter gene three novel compounds, cycloepoxydon (**1**), 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene (**2**) and 1-hydroxy-2-hydroxymethyl-3-pent-1,3-dienylbenzene (**3**) were isolated from fermentations of the deuteromycete strain *45–93*. Cycloepoxydon inhibits the TPA-induced NF-κB and AP-1 mediated SEAP expression with an IC_{50} of $1\sim2\,\mu g/ml$ ($4.2\sim8.4\,\mu M$) and $3\sim5\,\mu g/ml$ ($12.6\sim21\,\mu M$) respectively. 1-Hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene (**2**) inhibits the TPA-induced NF-κB and AP-1 mediated SEAP expression with an IC_{50} of $7\,\mu g/ml$ ($36.4\,\mu M$) and $5\,\mu g/ml$ ($26\,\mu M$). **3** showed only a weak inhibition of the AP-1 and no influence on NF-κB dependent reporter gene expression. In COS-7 and HeLa S3 cells electrophoretic mobility shift assays showed that cycloepoxydon strongly reduced the TPA and TNF-α mediated binding of NF-κB to a high affinity consensus sequence which was due to the inhibition of phosphorylation of the inhibitory protein IκB.

NF-κB is an inducible, ubiquitous transcription factor which regulates the expression of various cellular genes involved in immune response, inflammation, acute phase response, apoptosis and several viral genes[1]. NF-κB is composed of two subunits which belong to the Rel/NF-κB family of polypeptides. The most common form of NF-κB is a dimeric complex of p65 (RelA) and p50 (NF-κB1), but other homo- and heterodimers have been described and these different forms of NF-κB may have different regulatory effects[2]. In non-stimulated cells NF-κB is located in the cytoplasm complexed with an inhibitory protein IκB, of which several forms exist (IκB-α, IκB-β, IκB-γ, IκB-δ, IκB-ε)[1]. Much research has focused on the activation of NF-κB (p50/p65) bound to the inhibitory protein IκB-α. The inactive cytoplasmic NF-κB complex can be activated by stimulation of cells with a broad range of inducing agents like 12-*O*-tetradecanoylphorbol-13-acetate (TPA), TNF-α, ocadaic acid or cytokines. Upon stimulation IκB-α is phosphorylated by an inducible IκB kinase[3,4] which is followed by the ubiquitin-dependent degradation in the 26S proteasome[5]. This allows the translocation of the factor to the nucleus, DNA-binding of the active heterodimers composed of p50 and p65 subunits and the activation of transcription. Because NF-κB is involved as an immediate-early transcriptional activator of genes encoding pro-inflammatory cytokines, chemokines that selectively attract inflammatory cells, inflammatory enzymes (*e.g.* iNOS, COX-2) and adhesion molecules, inhibitors of NF-κB activation may therefore have broad application as novel therapeutics[6,7]. In addition, NF-κB operates in conjunction with other transcription factors and a synergistic interaction between NF-κB and the activator protein-1 (AP-1) in many inflammatory genes has been described[8,9]. Many stimuli including growth factors, cytokines, T cell activators and TPA that activate NF-κB also induce AP-1. Inhibitors specifically interfering with components of these pathways might be useful tools in studies of the underlying mechanism, as well as for the development of drugs against for example, cancer.

Fig. 1. Structures of cycloepoxydon (**1**), (**2**) and (**3**).



Cycloepoxydon (**1**)



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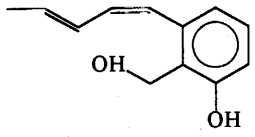

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In order to search for new inhibitors of the NF-κB and AP-1 mediated gene expression, COS-7 cells have been transiently transfected with reporter gene constructs, which contained the reporter gene (SEAP) under the control of five copies of the NF-κB binding consensus sequence or three copies of the TPA responsive element (TRE) respectively. A screening of some 200 strains of basidiomycetes, ascomycetes and fungi imperfecti resulted in the isolation of three compounds from the deuteromycete *45–93*. The structural elucidation of the compounds has been described elsewhere[10]. In this paper the fermentation of the producing strain, the isolation of cycloepoxydon (**1**), 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene 2 (**2**), 1-hydroxy-2-hydroxymethyl-3-pent-1,3-dienylbenzene (**3**) and the biological properties are described.

Materials and Methods

Producing Organism

The deuteromycete strain *45–93* was isolated as an infection of a *Xylaria* spec. (*Ascomycetes*). No conidia formation could be observed, the mycelia remained sterile. The strain is deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern.

Fermentation

For maintenance on agar slants and submerged cultivation, *45–93* was grown in YMG medium composed of (g/liter): glucose, 4; malt extract, 10; yeast extract, 4. Fermentations were carried out in a Biolafitte C-6 fermenter containing 20 liters of YMG medium with aeration (3 liters air/minute) and agitation (120 rpm) at 22°C. The inhibitory effect of various concentrations of a crude extract of the culture fluid was estimated in the reporter gene assay as described below. In addition, the production of cycolepoxydon could be followed by a plate diffusion assay using *Nematospora coryli* as test organism: 100 ml of fermentation broth were extracted with 100 ml of EtOAc, the organic phase evaporated and the residue dissolved in 1 ml of methanol. 10 µl of this solution were used in the plate diffusion assay.

Isolation

After 6 days the culture fluid (18 liters) was applied onto a column (4 × 40 cm) with Mitsubishi DIAION HP 21 resin. After washing with H_2O the active compounds were eluted with 2 liters of acetone.

The solvent was evaporated and the crude product (2.25 g) was separated by column chromatography on silica gel (Merck 60; 25 × 40 cm). After elution with cyclohexane - EtOAc (40 : 60 for cycloepoxydon and 80 : 20 for **2** and **3**) 360 mg crude extract 1 and 420 mg crude extract 2 were obtained. Cycloepoxydon was further purified from extract 1 by repeated column chromatography on silicic acid (Sigma) with cyclohexane - EtOAc (50 : 50) as eluant and crystallized from *tert*-butyl methyl ether yielding 1.9 mg of **1** per liter of culture fluid.

Compounds **2** and **3** were purified from extract 2 by preparative HPLC (Merck LiChrosorb Diol, 7 µm; column size 250 × 25 mm; flow rate 5 ml/minute) with a cyclohexane-*tert*-butyl methyl ether gradient (0 ~ 10 minutes: 0 ~ 40%; 10 ~ 90 minutes: 60 ~ 100%). Retention time for **2** was 41.6 minutes, for **3** 51.3 minutes.

Biological Assays

COS-7 (ATCC CRL 1651), HeLa S3 (ATCC CCL 2.2.) and BHK-21 cells (ATCC CCL 10) were maintained in DMEM-medium supplemented with 10% fetal calf serum (FCS) and 65 µg/ml penicillin G and 100 µg/ml streptomycin sulfate. L1210 (ATCC CCL 163) and HL-60 cells (ATCC CCL 240) were grown in RPMI 1640 medium with 10% FCS.

The assays for antimicrobial[11], cytotoxicity[12] as well as macromolecular syntheses[13] in COS-7 cells were carried out as described previously. Mutagenicity was tested as described by VENITT *et al.* (1984)[14]. Mutants of *Salmonella typhimurium*, strain TA 98 and TA 100 were used for the plate incorporation test with and

without rat liver microsomes.

Reporter gene assays: The reporter plasmids pGE2-NF1 and pGE2-AP1 were constructed as described by ERKEL *et al.* (1996)[15]. Both plasmids carry the reporter gene secreted alkaline phosphatase (SEAP) under the control of an enhancerless SV40 promoter and 5 × NF-κB or 3 × AP-1 binding sites.

Transfections of COS-7 cells were performed by electroporating (Bio-Rad Gene pulsor) 3×10^6 cells suspended in 1 ml phosphate buffered saline (PBS) together with 30 μg of the reporter constructs at 500 V/cm and $\tau = 20 \sim 23$ ms. The cells were appropriately diluted and seeded at 1×10^5 cells/ml in Opti-MEM medium containing 10% FCS. For induction of SEAP expression the cells were treated after 16 hours with 50 ng/ml TPA with or without test compounds in Opti-MEM containing 0.5% FCS. The activity of the SEAP in the culture medium was determined 60 hours after transfection using the Phospha-Light chemoluminescent reporter gene assay (TROPIX, MA) according to the manufacturer's instructions with a liquid scintillation counter.

Electrophoretic mobility shift assays (EMSAs): COS-7 and HeLa S3 cells were starved for 16 hours in DMEM-medium with 0.5% FCS, treated for 2 hours with test compounds and induced with 50 ng/ml TPA. Total cell extracts using a high-salt detergent buffer were prepared as described recently[16].

Westen Blot analysis: HeLa S3 cells were treated as described above and induced with 10 ng/ml TNF-α. From the total cell extracts 10 μg of protein were loaded in each lane of 10% SDS-polyacrylamide gel. Following electrophoretic transfer to ECL Hybond (Amersham International, UK) the membrane was probed with a anti- IκB-αntibody (Santa Cruz Biotechnology, CA) and then with a goat anti-rabbit antibody conjugated to horse radish peroxidase. The blots were detected by enhanced chemoluminescence (ECL System, Amersham International, UK).

Results

Production of cycloepoxydon (**1**) started 2~3 days after inoculation of fermentations of *45–93* (Fig. 2). The antifungal activity correlating to the cycloepoxydon content peaked at the end of the fermentation when all the glucose and maltose were used up. The isolation of cycloepoxydon was performed as described in the experimental section. In addition to cycloepoxydon smaller amounts of **2** and **3** were obtained from the same crude extract.

Fig. 2. Fermentation of *45–93* in 20 liters YMG medium.

○ pH; × mycelial dry weight (g/liter); △ maltose (g/liter); □ glucose (g/liter); * diameter of inhibition zone against *Nematospora coryli* as test organism (10 μl of crude extract).

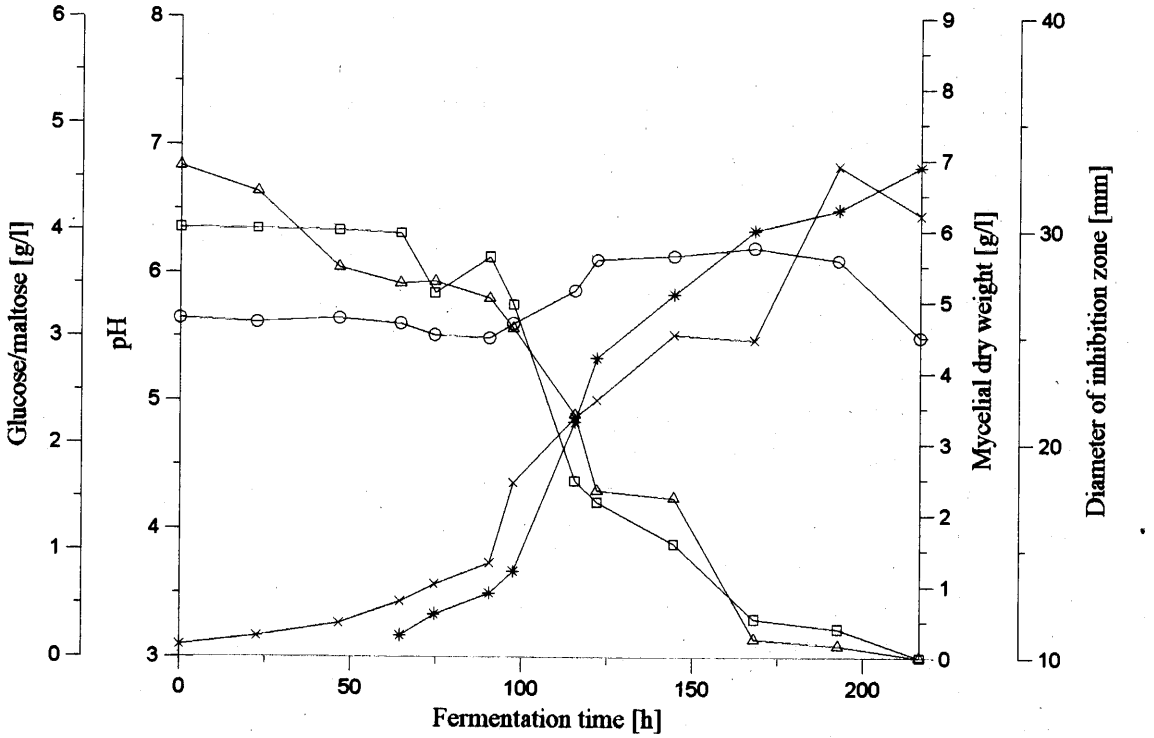
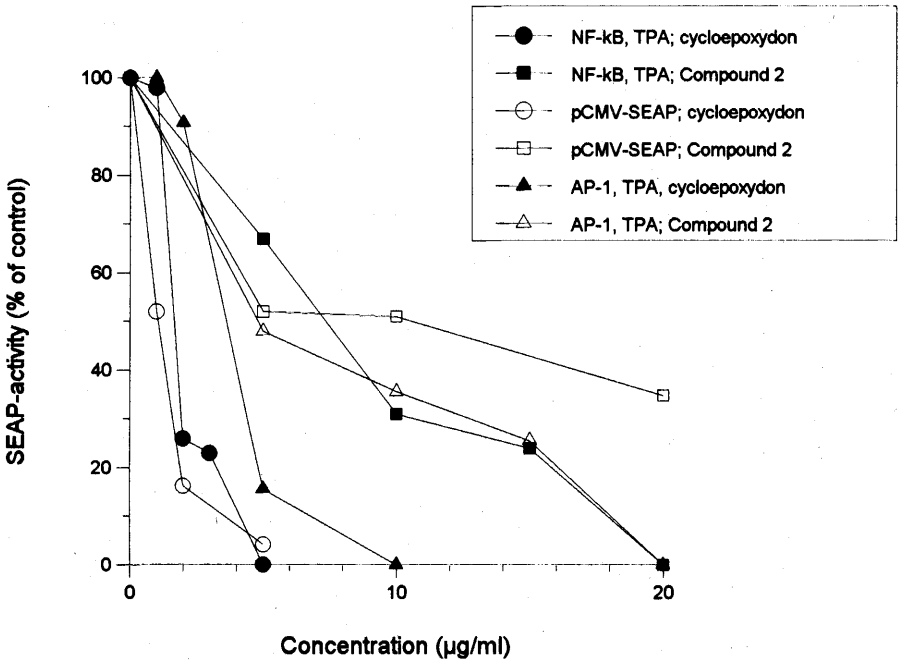


Fig. 3. Inhibition of NF-κB and AP-1 mediated SEAP expression in COS-7 cells by cycloepoxydon and compound **2**.



COS-7 cells were transfected with pCMV- SEAP or a reporter gene construct containing the SEAP gene under the control of the SV40 minimal promotor and 5 × NF-κB or 3 × AP-1 consensus sequences and stimulated with TPA (50 ng/ml) for 48 hours with or without the two compounds. Control (100%): stimulation only.

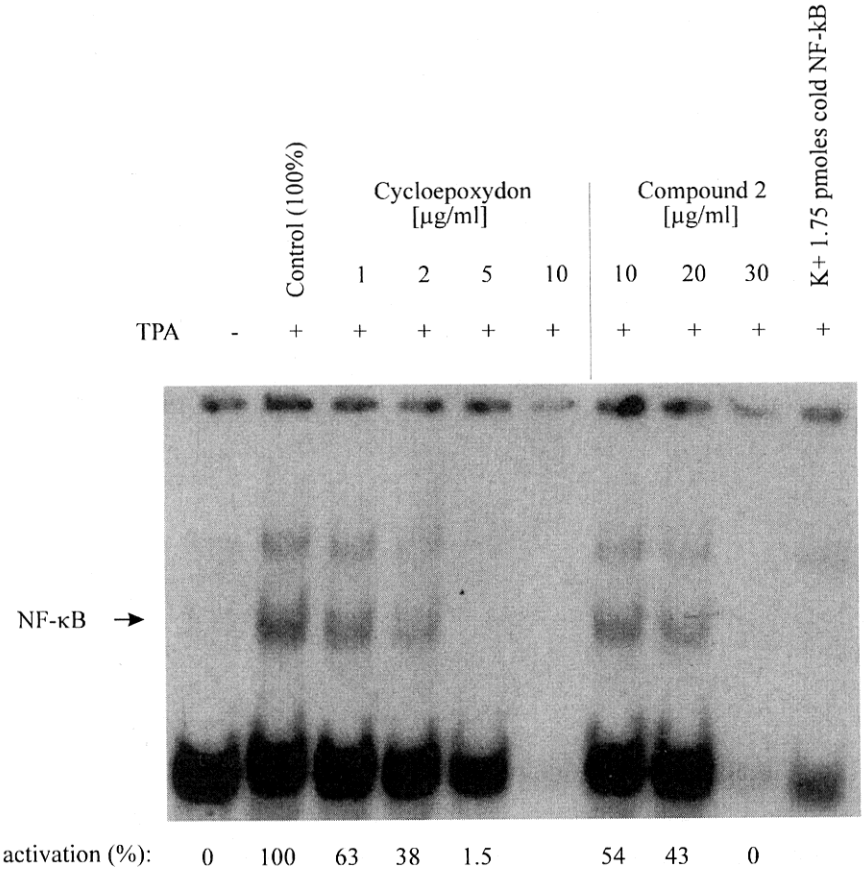
Biological Properties

The transfection of COS-7 cells with the SEAP reporter vectors pGE2-NF1 containing five copies of the NF-κB consensus sequence or pGE2-AP1 containing three copies of the TPA responsive element (TRE) and induction with TPA (50 ng/ml) resulted in a 20 fold (pGE2-NF1) or 8 fold (pGE2-AP-1) activation over the basal level of SEAP expression[15]. Cycloepoxydon inhibited the TPA induced NF-κB and AP-1 mediated SEAP expression in COS-7 cells with IC_{50} values of $1 \sim 2\,\mu g/ml$ ($4.2 \sim 8.4\,\mu M$) and $3 \sim 5\,\mu g/ml$ ($12.6 \sim 21\,\mu M$) respectively (Fig. 3). In addition, the SEAP expression in COS-7 cells, transfected with the pCMV-SEAP vector (Promega) which is regulated by the constitutive CMV-promotor was also inhibited to 50% by $4.2\,\mu M$ cycloepoxydon. This might be due to the occurrence of multiple enhancer sequences (including NF-κB and AP-1) in the CMV-promoter. The compound **2** inhibited the TPA-induced NF-κB and AP-1 mediated SEAP expression with an IC_{50} of $7\,\mu g/ml$ ($36.4\,\mu M$) and $5\,\mu g/ml$ ($26\,\mu M$). The effect on the SEAP expression regulated by the constitutive CMV-promoter was much less pronounced. **3** showed only weak inhibition of AP-1 mediated SEAP expression ($IC_{50} = 15\,\mu g/ml$, $78\,\mu M$) and was therefore not further tested.

Cellular DNA-, RNA-, and protein syntheses were examined in COS-7 cells by determing the incorporation of [^{14}-C]-thymidine, [^{14}-C]-uridine and [^{14}-C]-leucine into TCA-insoluble fractions. Up to a concentration of $20\,\mu g/ml$ of cycloepoxydon and $30\,\mu g/ml$ of **2** no effect on macromolecular syntheses could be observed during 30 minutes incubation suggesting that cycloepoxydon and **2** do not interfere with replication, transcription and translation in a general manner. In addition, the compounds cycloepoxydon and **2** showed antiproliferative properties. At concentrations starting from $5\,\mu g/ml$ of cycloepoxydon and $20\,\mu g/ml$ of **2** the growth of COS-7, HeLa S3, BHK-21, L1210 and HL-60 cells was almost completely blocked. The cells remained viable for at least two days. Both compounds exhibit only weak antibacterial activities starting from $50\,\mu g/ml$ and in the test for mutagenicity no induction of revertants of *S. typhimurium* TA 98 and TA 100 could be observed with $25\,\mu g/ml$ of cycloepoxydon and **2** (pour plate assay with and without addition of rat liver microsomes)[14].

In order to determine the effect of cycloepoxydon on activation of NF-κB in COS-7 cells, EMSA's with whole

Fig. 4a. Effect of cycloepoxydon on NF-κB activation in COS-7 cells.



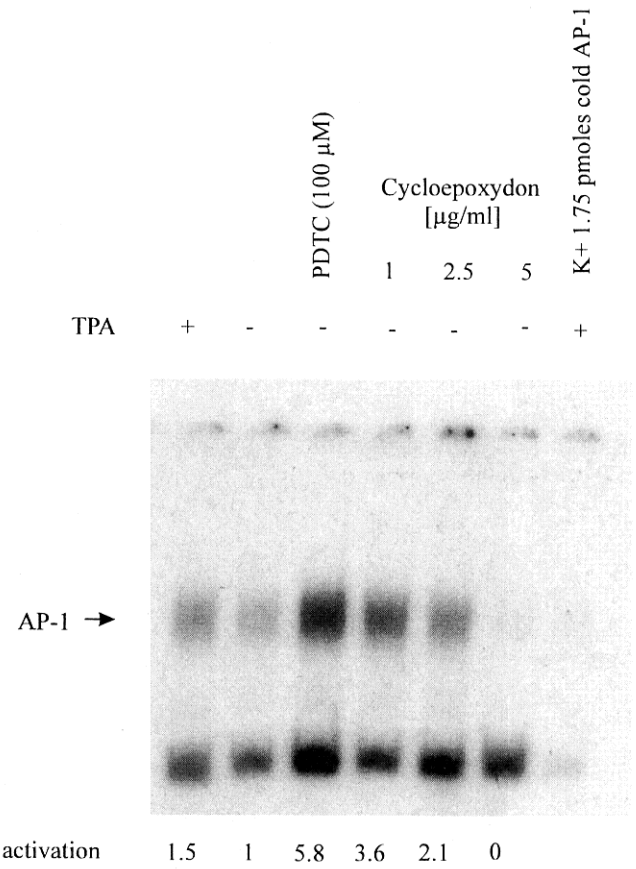
COS-7 cells were treated for 48 hours with TPA (50 ng/ml) without or with various concentrations of cycloepoxydon and **2**. Total cell extracts containing equal amounts of protein (10 μg) were analyzed by EMSA for DNA binding activity of NF-κB using a ^{32}P-labelled oligonucleotide with the high affinity site 5′ AGTTGAGGGGACTTTCCCAGGC-3′. The inducible NF-κB complex is indicated. A section of the fluorogram from a native gel is shown.

cell extracts were performed with a high affinity binding site. Stimulation of the cells with 50 ng/ml TPA for 48 hours resulted in the appearance of a band which disappeared after addition of a 10 fold excess of unlabelled NF-κB probe. (Fig. 4a). Addition of cycloepoxydon and **2** resulted in a reduction of the NF-κB binding activity in a dose dependent manner. The NF-κB activation was inhibited by cycloepoxydon and **2** to 50% at 4.2～8.4 μM and 52～104 μM respectively (quantification as percentage of the fully induced control was done by densiotmetry scanning). These results correspond well to the ones obtained from the reporter gene assays. EMSA's of the same cell extracts (after stimulation with 50 ng/ml TPA for 48 hours) with a high affinity binding site for AP-1 revealed an inhibition of AP-1 binding activity at 21 μM for cycloepoxydon and 104 μM **2** (data not shown) which might be due to the cytostatic effects of the compounds.

EMSA's with whole cell extracts from HeLa S3 cells were performed after stimulation with 50 ng/ml TPA for 48 hours. Pretreatment of HeLa S3 cells with 5 μg/ml (21 μM) of cycloepoxydon for 2 hours before stimulation showed a similar reduction in NF-κB binding activity as could be observed in COS-7 cells (data not shown).

The release of IκB-α leading to an active NF-κB-complex requires phosphorylation of the IκB-α protein on serines 32 and 36 and the subsequent proteolytic degradation by the ubiquitin/proteasome pathway. The inducible phosphorylated form of IκB-α can be detected by a slight decrease of mobility in SDS gels[16,17]. Proteasome inhibitors like PSI (Cbz-Ile-Glu(*O*-*t*-Bu)-Ala-leucinal) or *N*-acetyl-Leu-Leu-norleucinal (Ac-LLnL) inhibit the activation of NF-κB by accumulation of the phosphorylated form of IκB-α which results in an inactive complex. To investigate whether cycloepoxydon

Fig. 4b. Effect of cycloepoxydon on AP-1 activation in COS-7 cells.

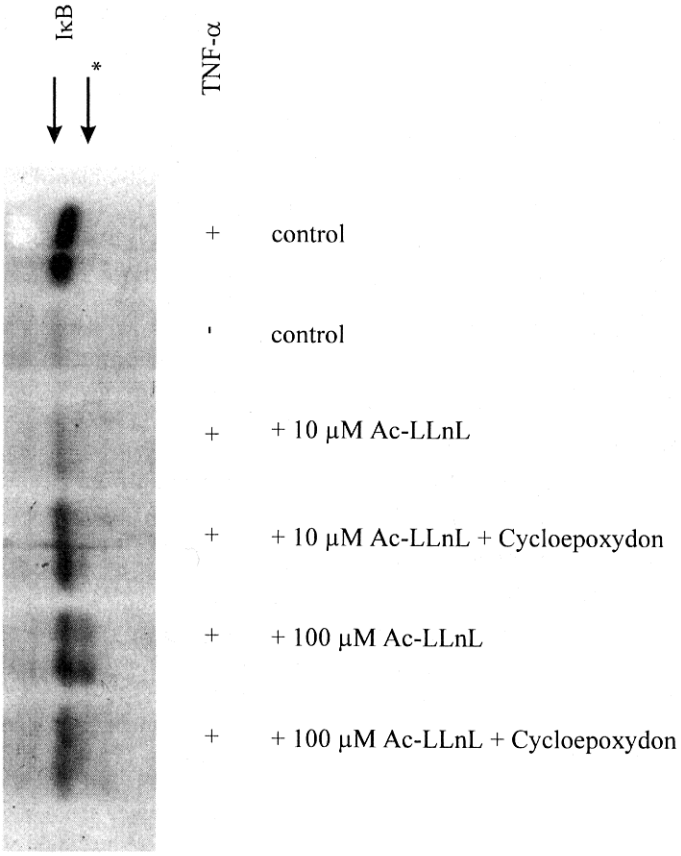


COS-7 cells were treated for 24 hours with various concentrations of cycloepoxydon. Total cell extracts containing equal amounts of protein (10 μg) were analyzed by EMSA for DNA binding activity of AP-1 using a ^{32}P-labelled oligonucleotide. The inducible AP-1complex is indicated. A section of the fluorogram from a native gel is shown.

prevented NF-κB activation by inhibiting the phosphorylation of IκB, HeLa S3 cells were left untreated or pretreated for 2 hours with 10 and 100 μM of the peptide inhibitor Ac-LLnL and stimulated with 10 ng/ml TNF-α for 20 minutes. Afterwards total cell extracts were immunoblotted with an IκB antibody. The stabilization of the hyperphosphorylated form of IκB can be seen in Fig. 5. After pretreatment of HeLa S3 cells with Ac-LLnL in the presence of 2 μg/ml cycloepoxydon and stimulation with TNF-α, no stabilization of the phosphorylated form of IκB could be observed (Fig. 5). These results indicate that cycloepoxydon inhibits NF-κB activation by preventing the phosphorylation of IκB leading to an inactive NF-κB complex.

The activation of both NF-κB and AP-1 seem to be regulated by redox-sensitive signaling pathways. NF-κB is an oxidant-inducible factor whose activation can be inhibited by various antioxidants *e.g.* *N*-acetyl-L-cysteine (NAC), vitamin E derivatives, 2-mercaptoethanol, dithiocarbamates, butylated hydroxyanisol, α-lipoic acid and chelators of iron and copper ions. The DNA-binding activity of AP-1 is regulated by redox modification of conserved cysteine residues in the DNA-binding domain of *c-jun* and *c-fos*. In addition, the transactivation and the DNA-binding activity of AP-1 are induced with thioredoxine or the antioxidants pyrrolidine dithiocarbamate (PDTC) and NAC. Therefore both transcription factors seem to be regulated oppositely by redox-dependent processes[18,19]. To investigate whether cycloepoxydon alone could induce DNA binding of the transcription factor AP-1 complex, COS-7 cells were treated with various concentrations of cycloepoxydon for 24 hours and EMSA's with whole cell extracts were performed with a high affinity binding site for AP-1. Treatment of the cells with 1$\sim$2 μg/ml cycloepoxydon led to a 2$\sim$3 fold stimulation of DNA binding of the AP-1 transcription factor complex as compared to the uninduced control. (Fig. 4b). At 5 μg/ml the disappearance

Fig. 5. Effect of cycloepoxydon on the stability of IκB in HeLa S3 cells.



HeLa S3 cells were left untreated or pretreated for 2 hours with 10 μM or 100 μM Ac-LLnL with or without 2 μg/ml cycloepoxydon and stimulated with 10 ng/ml TNF-α for 20 minutes. Total cell extracts were prepared and western blotting was performed as described under Materials and Methods using an IκB antibody. The asterik marks the more slowly migrating phosphorylated IκB variant.

of the band seemed to be the result of cytostatic effects of cycloepoxydon. TPA (50 ng/ml, 1.5 fold activation) and the antioxidant PDTC (100 μM, 5.8 fold activation) were used as positive controls.

Discussion

Cycloepoxydon (**1**), 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene (**2**) and 1-hydroxy-2-hydroxymethyl-3-pent-1,3-dienylbenzene (**3**) are three new hexaketides which inhibit the NF-κB and the AP-1 mediated signal transduction in COS-7 cells. Whereas compounds **2** and **3** showed no selective inhibition for one of the transcription factors, cycloepoxydon selectively inhibited the activation of NF-κB by preventing the phosphorylation of the IκB protein. In addition, the inhibitory effect of cycloepoxydon on NF-κB induction seems also to be partially due to an antioxidant mode of action since an activation of the transcription factor AP-1 could be observed at concentrations (4.2~8.4 μM) where NF-κB was inhibited. The same effect has also been described for the antioxidant PDTC[18,20]. Recently a structurally related compound, panepoxydone, has been isolated from *Lentinus crinitus*[15]. Like cycloepoxydon, panepoxydone strongly inhibited the activation of the NF-κB transcription factor by preventing the phosphorylation of the IκB protein but no stimulation of an AP-1 reporter gene construct and the DNA binding activity of the transcription factor AP-1 could be observed. The mycotoxin gliotoxin also inhibits the activation of NF-κB by preventing the degradation of the inhibitory protein IκB and it has been suggested that the immunosuppressive activity of gliotoxin results in part from the inhibition of NF-κB[20].

It has been shown that many stimuli like inflammatory cytokines, TNF, LPS, TPA and PDTC activate the stress-responsive transcription factor AP-1, which comprises a homo- or heterodimeric complex composed of

the Fos, Jun and ATF families of transcription factors, which display different transcriptional activities[21,22]. Various stress activated kinases like c-jun NH_2-terminal kinase (JNK) and p38 are involved in the activation of AP-1 and in turn increase the *c-jun* and *c-fos* expression[23]. Activation of JNK and p38 by antioxidants has been observed in different cell lines whereas the activation of NF-κB by all stimuli analyzed so far is inhibited by antioxidants[2,24,25]. Our findings suggest that cycloepoxydon activates the binding of the transcription factor AP-1 to its consensus sequence through the stimulation of the stress response pathways at least partially via an antioxidant mode of action. On the other hand we have shown that the inhibition of NF-κB activation is also due to the inhibition of the phosphorylation of the IκB protein. Furthermore it seems conceivable that the inhibition of an AP-1 dependent reporter gene, after stimulation with the phorbol ester TPA (which activates the extracellular signal-regulated kinases ERK 1,2)[26,27] is caused by the interference of cycloepoxydon with one or several kinases involved in this signaling pathway. The exact mode of action of cycloepoxydon on these different cellular targets remains to be examined.

Acknowledgements

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