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# Structural Requirements of Sesquiterpene Lactones to Inhibit LPS-Induced Nitric Oxide Synthesis in RAW 264.7 Macrophages

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**Abstract**—Some sesquiterpene lactones were recently demonstrated to inhibit inducible nitric oxide synthase (iNOS)-dependent nitric oxide (NO) synthesis. The primary objective of the present study was, therefore, to find evidence for structural requirements of sesquiterpene lactones regarding their capability to inhibit iNOS-dependent NO synthesis. Sesquiterpene lactones **1**–**11** were examined for their influence on nitrite accumulation in cell culture supernatants of LPS-induced RAW 264.7 macrophages. Except the taraxinic acid β-D-glucopyranosylester **8** all compounds showed a dose-dependent inhibition of nitrite accumulation in cell culture supernatants with $IC_{50}$ values ranging from 0.5 to 36.8 μM. High activity seemed to be dependent on an α-methylene-γ-lactone functionality. Cytotoxicity and the ability to inhibit activation of transcription factor NF-κB are further biological activities of sesquiterpene lactones. The second point of interest was, therefore, whether the structural requirements of sesquiterpene lactones for these activities may differ or be the same for those needed to inhibit iNOS-dependent NO synthesis. Using concentrations of **1**–**11** required to inhibit NO synthesis cell viability was determined and NF-κB binding activity was measured by gel-shift experiments. Interestingly, compounds almost equally effective in inhibiting nitrite accumulation did not show the same cytotoxic potential, and most sesquiterpene lactones inhibited nitrite accumulation at concentrations where inhibition of NF-κB activation was not significant. These results suggest that different biological activities of sesquiterpene lactones have different structural requirements.


## Introduction

Sesquiterpene lactones are biologically active natural products found almost exclusively in species of the Compositae (Asteraceae) family. Common structural features of this class of compounds are a terpenoid C-15 skeleton and a γ-lactone moiety. Other structural elements can vary considerably. Depending on the basic skeleton, different subgroups are distinguished, e.g., germacranolides, eudesmanolides, guaianolides and pseudoguaianolides. Pharmacological activities described for sesquiterpene lactones include antimicrobial,[1] antiviral,[2] cytotoxic or antitumor,[1,3] and anti-inflammatory properties.[4–8] Recently, several sesquiterpene lactones, i.e., parthenolide, isohelenin, dehydrocostus lactone, and yomogin, were shown to inhibit the expression of inducible nitric oxide synthase (iNOS) in various cell systems.[9–11] iNOS is an enzyme which is induced in different cell types by pro-inflammatory stimuli.[12] Excessive production of nitric oxide (NO) by this enzyme is considered as a promoter of tissue injury in inflammation. iNOS is, therefore, an important target involved in inflammatory and also immunoregulatory processes.[13] The ability of sesquiterpene lactones to modulate iNOS expression is suggested to contribute to the anti-inflammatory properties of these compounds.[9]

In this context, studies aimed at elucidating the structural requirements of sesquiterpene lactones regarding their potential to inhibit iNOS-dependent NO synthesis are important to find new potent inhibitors. The primary objective of this study was, therefore, to investigate sesquiterpene lactones from different structural subgroups for their potential to inhibit iNOS-dependent NO synthesis in order to find structural requirements for this biological activity.

So far, the best characterized biological activities of sesquiterpene lactones are their cytotoxicity[1,3,14,15] and

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the ability of sesquiterpene lactones to inhibit the activation of the transcription factor nuclear factor kappaB (NF-κB).[5–9] Both activities are of interest in the context of the present study: in vitro cytotoxicity may simulate inhibition of NO synthesis and is undesirable from a pharmacological point of view. Activation of NF-κB was shown to be necessary for iNOS expression.[16] Moreover, Wong et al. provided evidence that sesquiterpene lactones inhibit iNOS expression via inhibition of NF-κB.[9] Therefore, our second point of interest was to learn whether the structural requirements of sesquiterpene lactones needed for the inhibition of nitric oxide synthesis correlate with those necessary for their cytotoxic potential and their ability to inhibit activation of NF-κB.

For these studies RAW 264.7 macrophages activated by bacterial endotoxic lipopolysaccharide (LPS) were chosen since induction of iNOS was shown to be dependent on activation of NF-κB in this cell system.[16] Sesquiterpene lactones **1**–**11** (Chart 1) were selected from different structural subgroups. First, the influence of **1**–**11** on iNOS-dependent NO synthesis was determined by measuring nitrite accumulation in cell culture super-

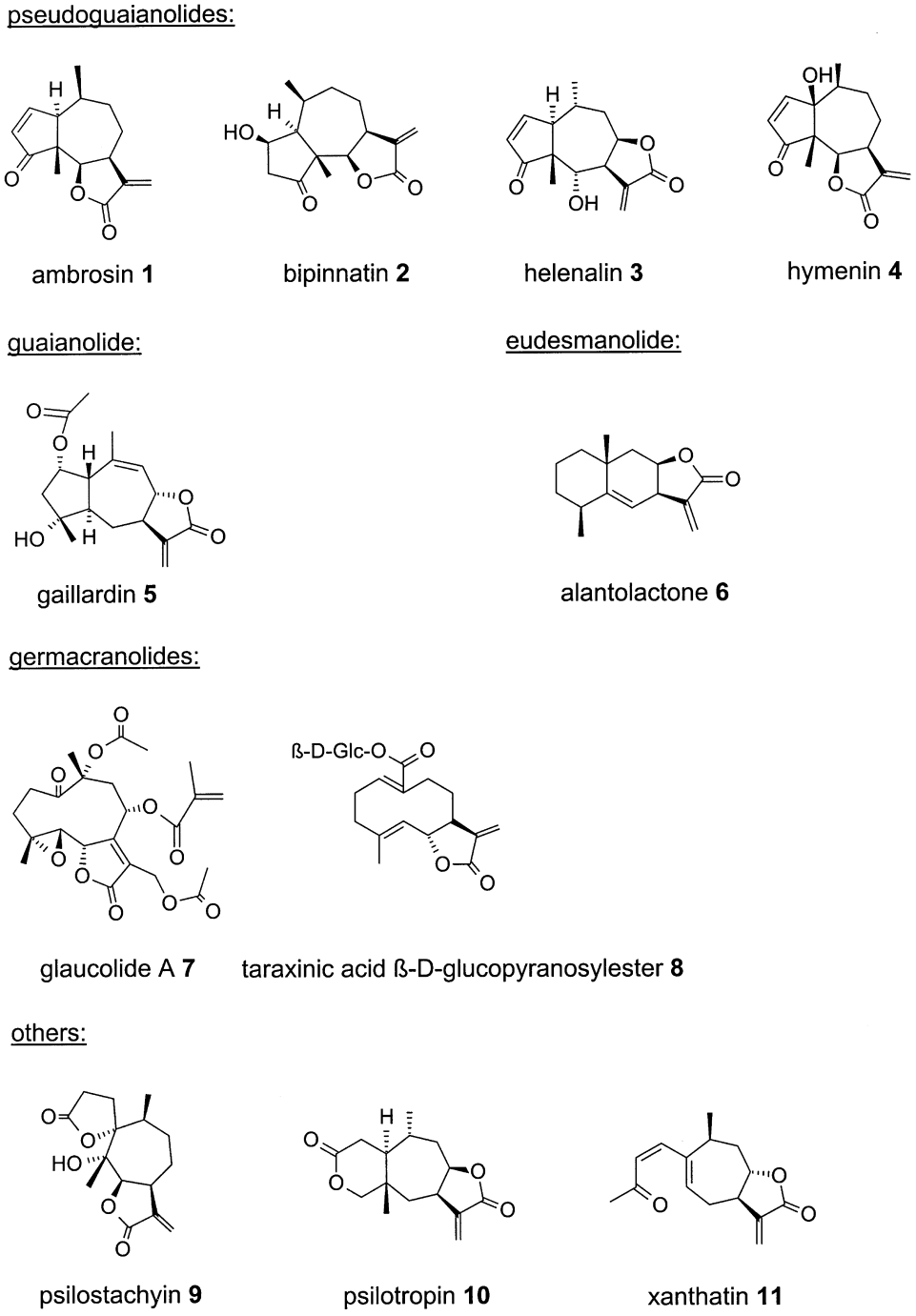


**Chart 1.** Sesquiterpene lactones **1**–**11**.

natants. Second, using the determined effective concentrations of **1**–**11** the influence on cell viability and NF-κB activation was measured.

## Results

### Inhibition of LPS-induced nitrite accumulation in RAW 264.7 cells by sesquiterpene lactones 1–11. Structural requirements

In order to investigate whether sesquiterpene lactones **1**–**11** are able to inhibit inducible nitric oxide synthase (iNOS)-dependent nitric oxide (NO) synthesis, we determined nitrite accumulation (20 h) in cell culture supernatants of lipopolysaccharide (LPS)-activated (1 μg/mL) and sesquiterpene lactone-treated RAW 264.7 macrophages seeded in 96-well plates. Maximum concentration of sesquiterpene lactones **1**–**11** employed was 100 μM. Table 1 shows that indeed all non-glycosylated sesquiterpene lactones were able to inhibit NO synthesis with $IC_{50}$ values far below 100 μM. The highest activity ($IC_{50} < 1$ μM) was found for hymenin **4** ($IC_{50}$ 0.5 μM), alantolactone **6** ($IC_{50}$ 0.6 μM) and helenalin **3** ($IC_{50}$ 0.9 μM), followed by compounds with $IC_{50}$ values between 1 and 10 μM, namely ambrosin **1** ($IC_{50}$ 3.2 μM), bipinnatin **2** ($IC_{50}$ 3.3 μM), xanthatin **11** ($IC_{50}$ 5.0 μM), psilotropin **10** ($IC_{50}$ 6.2 μM) and psilostachyin **9** ($IC_{50}$ 8.7 μM). $IC_{50}$ values $> 10$ μM were determined for gaillardin **5** ($IC_{50}$ 12.2 μM), glaucolide A **7** ($IC_{50}$ 36.8 μM) and taraxinic acid β-D-glucopyranosylester **8** ($IC_{50} > 100$ μM). All effects were dose-dependent (data not shown).

The biological activity of sesquiterpene lactones was suggested to be mediated by the reaction of α-methylene-γ-lactones and other conjugated systems with sulfhydryl groups. For various activities the α-methylene-γ-lactone moiety seems to be the most important functional group. Other α,β-unsaturated carbonyl structure elements were reported to enhance these activities.[6,14,15] We, therefore, tried to find a correlation between the potential of sesquiterpene lactones **1**–**11** to inhibit iNOS-dependent NO synthesis and their number of reactive α,β-unsaturated carbonyl structure elements. Table 1 shows that there is no direct correlation between the number of potentially active centers of sesquiterpene lactones and their NO inhibitory activity. Even within the group of pseudoguaianolide-type sesquiterpene lactones ambrosin **1** ($IC_{50}$ 3.2 μM, two active centers) shows the same activity as bipinnatin **2** ($IC_{50}$ 3.3 μM, one active center). However, the combination of a lactone ring with a conjugated exomethylene group (α-methylene-γ-lactone) seems to be important, since glaucolide A **7**, the only sesquiterpene lactone missing the exomethylene group in the lactone ring, showed the weakest activity ($IC_{50}$ 36.8 μM) of all non-glycosylated sesquiterpene lactones investigated (Table 1). To support the idea that the α-methylene-γ-lactone moiety might be essential for the NO inhibitory activity, we hydrogenated the double bonds in alantolactone **6**. Alantolactone **6** was chosen since it was the most active constituent bearing as the only reactive functional element the α-methylene-γ-lactone. Indeed, we found that the obtained mixture (the dihydroalantolactone lacking the exocyclic methylene group and the tetrahydro-alantolactone in a ratio of 45:55) showed no significant activity up to 50 μM (data not shown).

Glycosylation seems to negatively modulate activity, as seen by taraxinic acid-β-D-glucopyranosylester **8** (Table 1).

### Influence of 1–11 on cell viability in comparison to inhibition of nitrite accumulation

Viability of cells was determined after removing cell culture supernatants for nitrite measurement, i.e., after an incubation time of 20 h. As seen in Table 1, no sesquiterpene lactone impaired cell viability at the concentration determined as $IC_{50}$ value regarding inhibition of nitrite accumulation. However, all sesquiterpene lactones effective in inhibiting nitrite accumulation showed cytotoxicity at some higher concentrations. Interestingly, compounds almost equally effective in inhibiting

**Table 1.** $IC_{50}$ values obtained from measuring nitrite accumulation, cell viability and structural functionality of **1**–**11**

| Compound | $IC_{50}$ value (μM) | Unimpaired cell viability observed until (μM) | Mono- or bi-functional | α-Methylene-γ-lactone | Sugar moiety |
|---|---|---|---|---|---|
| Hymenin **4** | 0.5 | 10 | bi (ml[a]; cp[b]) | ✓ | |
| Alantolactone **6** | 0.6 | 2 | mono (mL) | ✓ | |
| Helenalin **3** | 0.9 | 2 | bi (ml; cp) | ✓ | |
| Ambrosin **1** | 3.2 | 5 | bi (ml; cp) | ✓ | |
| Bipinnatin **2** | 3.3 | 50 | mono (mL) | ✓ | |
| Xanthatin **11** | 5.0 | 25 | bi (ml; uc[c]) | ✓ | |
| Psilotropin **10** | 6.2 | 50 | mono (mL) | ✓ | |
| Psilostachyin **9** | 8.7 | 50 | mono (mL) | ✓ | |
| Gaillardin **5** | 12.2 | 50 | mono (mL) | ✓ | |
| Glaucolide A **7** | 36.8 | 75 | bi (uc; ep[d]) | | |
| Taraxinic acid-β-D-glucopyranosylester **8** | > 100 | 100 | bi (ml; uc) | ✓ | ✓ |

[a]α-Methylene-γ-lactone.
[b]α,β-Unsubstituted cyclopentenone.
[c]α,β-Unsaturated carbonyl structure.
[d]Epoxide.

nitrite accumulation, did not show the same cytotoxic potential, e.g., helenalin **3** ($IC_{50}$ 0.9 μM) showed quite profound cytotoxicity at a concentration as low as 5 μM (viability 53%, $n=4$; SEM: 0.023), whereas the even more effective hymenin **4** ($IC_{50}$ 0.5 μM) did not show cytotoxicity up to 10 μM. Similarly, ambrosin **1** ($IC_{50}$ 3.2 μM) showed first signs of cytotoxicity at a concentration of 10 μM (viability 91%, $n=3$; SEM: 0.024), whereas bipinnatin **2** ($IC_{50}$ 3.3 μM) showed no cytotoxicity at all until 50 μM.

### Influence of 1–11 on NF-κB binding activity in comparison to inhibition of nitrite accumulation

All sesquiterpene lactones except the less effective glycosylated **8** were examined for their influence on NF-κB binding activity in LPS-activated RAW 264.7 cells by the electrophoretic mobility shift assay (EMSA). Concentrations employed in this assay were within the determined non-toxic range and above the ascertained $IC_{50}$ values regarding nitrite accumulation (Table 1). To obtain enough nuclear protein for EMSA experiments we had to switch from cell culturing in 96-well plates to 6-well plates. Since different cell culture conditions may affect experimental results, i.e., results obtained in 6-well plates might differ from those obtained in 96-well plates, all EMSA experiments were set up in parallel for measuring nitrite accumulation. As a positive control we used PDTC (pyrrolidinedithiocarbamate), a known inhibitor of NF-κB activation and iNOS induction. A representative gel-shift experiment is shown in Figure 1. Experiments, each performed at least three times, were quantified by densitometry and the results compared to values obtained in parallel from measuring nitrite accumulation. As can be seen in Figure 2, most sesquiterpene lactones applied at concentrations significantly inhibiting iNOS-dependent NO synthesis do not show a significant inhibition of NF-κB activation. Only psilotropin **10** and gaillardin **5** are able to reduce iNOS-dependent NO synthesis and NF-κB activation to a similar extent.

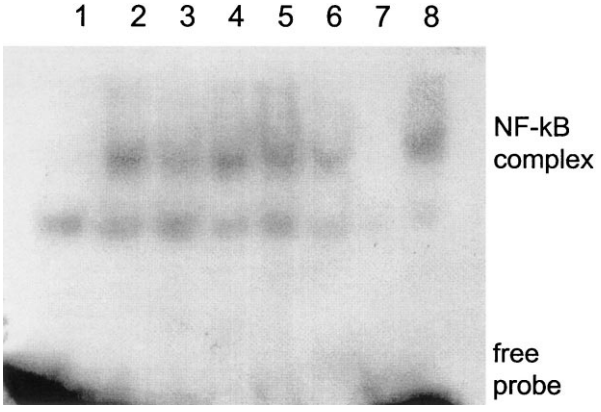


**Figure 1.** Representative gel-shift experiment showing the effect of sesquiterpene lactones ambrosin **1** (5 μM), bipinnatin **2** (5 μM), and gaillardin **5** (20 μM) as well as the positive control PDTC (50 μM) on the NF-κB binding activity in nuclear extracts of RAW 264.7 cells activated with LPS (1 μg/mL) for 1 h. Equal amounts of nuclear extracts (10 μg) were subjected to EMSA as described in the Experimental. Lane (1) untreated cells; lane (2) cells activated with LPS; lane (3) cells pretreated with PDTC (50 μM) for 2 h and then activated with LPS; lanes (4)–(6) cells pretreated with ambrosin **1** (5 μM, lane 4), bipinnatin **2** (5 μM, lane 5), gaillardin **5** (20 μM, lane 6) for 1 h and then activated with LPS; lane (7) nuclear extract of LPS-activated cells incubated with a 100-fold excess of unlabeled NF-κB consensus oligonucleotide (unlabeled competitor); lane (8) nuclear extract of LPS-activated cells incubated with a 100-fold excess of unlabeled AP-2 consensus oligonucleotide (unlabeled non-competitor).

Interestingly, PDTC (50 μM), which was shown to act not only as inhibitor of NF-κB activation but also as effective scavenger of NO formation,[17] inhibited nitrite accumulation more markedly (−84.2%, $n=8$, SEM: 2.183) than NF-κB activation (−54.5%, $n=8$, SEM: 9.272) in LPS-activated RAW 264.7 cells (Fig. 2).

## Discussion

The present study demonstrates that (i) all sesquiterpene lactones tested, except the glycosylated **8**, are able to inhibit iNOS-dependent nitrite accumulation dose-dependently with $IC_{50}$ values ranging from 0.5 μM to 36.8 μM; (ii) a high inhibitory activity correlated with the existence of an α-methylene-γ-lactone moiety but not necessarily with a bi-functionality of the respective compound; (iii) the inhibitory activities were not due to cytotoxicity. The cytotoxic potential observed at higher concentrations did not necessarily correlate with the ability to inhibit NO synthesis; (iv) most sesquiterpene lactones inhibited iNOS-dependent nitrite accumulation at concentrations where inhibition of NF-κB activation was not significant. The potency of sesquiterpene lactones to inhibit activation of NF-κB did not show a general correlation to the ability to inhibit NO synthesis.

The report that some sesquiterpene lactones inhibit iNOS-dependent nitric oxide synthesis[9–11] raised the question whether the ability to inhibit iNOS-dependent NO synthesis may be a common property of sesquiterpene lactones and which structural requirements might be necessary for this activity. We demonstrated that, indeed, a broad range of structural diverse constituents **1**–**11** were able to block LPS-induced NO synthesis in RAW 264.7 cells with different efficacy. Comparisons regarding structure–activity relationships showed that all sesquiterpene lactones with comparatively high activities ($IC_{50}<15$ μM) contained a conjugated exomethylene group in their lactone ring (α-methylene-γ-lactone). Glaucolide A **7**, the only sesquiterpene lactone lacking this α-methylene-γ-lactone group, was despite other potentially reactive elements (α,β-unsaturated carbonyl groups, epoxide group) less active than all other non-glycosylated sesquiterpene lactones investigated. The functional significance of the α-methylene-γ-lactone moiety was supported testing the mixture of diastereomers obtained upon hydrogenation of alantolactone **6**. Alantolactone bearing an α-methylene-γ-lactone was found to be the most active mono-functional sesquiterpene lactone ($IC_{50}$ 0.6 μM). After hydrogenation, the obtained mixture showed no significant effect on nitrite accumulation up to 50 μM. The observed impact of an α-methylene-γ-lactone group is in accordance with studies examining other biological activities

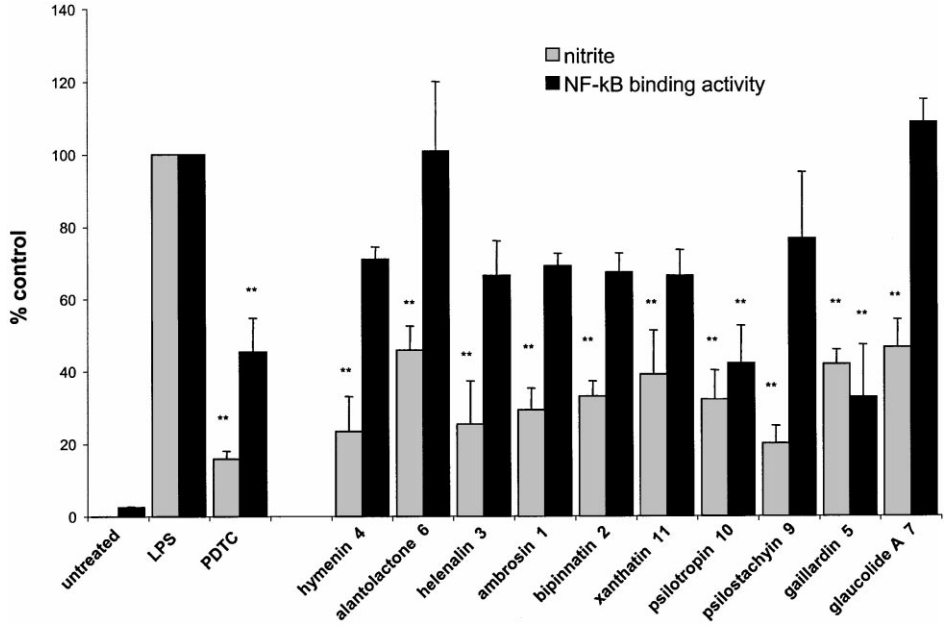


**Figure 2.** Comparison of the ability of sesquiterpene lactones **1**–**7** and **9**–**11** to inhibit nitrite accumulation in cell culture supernatants of LPS (1 μg/mL)-stimulated RAW 264.7 macrophages and NF-κB binding activity in nuclear extracts of RAW 264.7 cells activated with LPS (1 μg/mL) for 1 h. Values are given in % of the nitrite concentration and NF-κB binding activity detected in cell culture supernatants or nuclear extracts, respectively, of cells activated with LPS (100%). Employed concentrations: PDTC 50 μM, hymenin 2 μM, alantolactone 2 μM, helenalin 3 μM, ambrosin 5 μM, bipinnatin 5 μM, xanthatin 10 μM, psilotropin 10 μM, psilostachyin 15 μM, gaillardin 20 μM, glaucolide A 50 μM. Bars represent mean (±SEM) of at least three independent experiments. Nitrite measurement was performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ (ANOVA/Dunnett).

of sesquiterpene lactones: Kupchan et al. reported that the presence of an α-methylene-γ-lactone is essential for significant cytotoxic activity of sesquiterpene lactones.[14] Accordingly, investigations regarding the structural requirements for the inhibitory activity of sesquiterpene lactones on NF-κB activation found that compounds lacking this moiety display no inhibitory effect on activation of NF-κB.[8] On the other hand, other functional elements such as an α,β-unsaturated carbonyl structure or an epoxide group were suggested to enhance biological activity of sesquiterpene lactones.[5,6,14] Our results do not fit into this notion: as Table 1 demonstrates there was no obvious correlation between the $IC_{50}$ values and a mono- or bi-functionality. For example, ambrosin **1** containing an α,β-unsubstituted cyclopentenone in addition to the α-methylene-γ-lactone moiety inhibited nitrite accumulation equally to bipinnatin **2** with an α-methylene-γ-lactone only.

As observed previously studying glycosidic and non-glycosidic hypocretenolides,[17] a lipophilic character seems to be advantageous for high inhibitory activity in vitro. The fact that the taraxinic acid β-D-glucopyranosylester **8** exerted activity only higher than 100 μM, and the lipophilic alantolactone **6** was, despite its monofunctionality, one of the most active compounds is in line with this assumption.

The data presented here show that the structural requirements of sesquiterpene lactones for an effective inhibition of iNOS-dependent NO synthesis do not correspond to those reported in the literature for other biological activities, i.e., especially their cytotoxicity and the ability to inhibit activation of NF-κB.[5,6,14] We, therefore, performed our own experiments regarding these two well documented effects of sesquiterpene lactones comparing inhibition of nitric oxide synthesis, cytotoxicity, and inhibition of NF-κB in the same cell system.

Interestingly, we found that compounds with similar efficacy regarding inhibition of nitrite accumulation did not necessarily show the same cytotoxic activity when applied at the same concentration. For instance, hymenin **4** and bipinnatin **2** were found to be less cytotoxic than helenalin **3** and ambrosin **1**, respectively. Thus, regarding a potential therapeutic use, hymenin and bipinnatin are preferable. The different cytotoxic potentials of bipinnatin **2** and ambrosin **1** may be explained by an additional α,β-unsubstituted cyclopentenone in the ambrosin structure **1** compared to bipinnatin **2** with an α-methylene-γ-lactone element only.[14]

Measuring activation of NF-κB by gel-shift experiments, we found that most sesquiterpene lactones were more effective in inhibiting iNOS-dependent NO synthesis than NF-κB activation. These data suggest that there might be an additional mechanism, other than the sole inhibition of NF-κB, involved in the process leading to sesquiterpene lactone mediated reduction of NO synthesis. The antioxidant PDTC, which also inhibited nitrite accumulation more markedly than NF-κB activation (Fig. 2), was shown to act not only as inhibitor of NF-κB activation but also as effective scavenger of NO formation.[18] This mechanism cannot be excluded for sesquiterpene lactones. Conceivable additional mechanisms of action for sesquiterpene lactones might include an inhibition of the enzymatic activity of iNOS or interferences on other levels of iNOS regulation.

Furthermore, comparing data from nitrite measurement and gel-shift assays, we found that there was no general correlation for the potency of sesquiterpene lactones to inhibit NO synthesis and activation of NF-κB (Table 1), supporting the idea that the structural determinants for both activities might not be completely identical.

## Conclusion

We conclude that the most important structural requirement for inhibition of iNOS-dependent NO synthesis seems to be the α-methylene-γ-lactone moiety. Additional other functional elements like, e.g., an α,β-unsubstituted cyclopentenone seem to be less important. Data from the literature as well as our experiments support the idea that different biological activities of sesquiterpene lactones seem to have different structural requirements. However, the α-methylene-γ-lactone moiety seems to play a crucial role for most activities described so far. These data may provide a chance to find compounds which inhibit iNOS-dependent NO synthesis without cytotoxic potential.

## Experimental

### Materials

Helenalin and psilotropin were isolated from *Psilostrophe cooperi* (A. Gray) Green, ambrosin, hymenin, and bipinnatin from *Hymenoclea salsola* Torr. & A. Gray, and taraxinic acid-β-D-glucopyranosylester from *Taraxacaum linearisquameum* Soest; alantolactone, gaillardin, glaucolide A, psilostachyin, and xanthatin were a kind gift from Dr. E. Rodriguez (Cornell University, Ithaca, NY).

### Hydrogenation of alantolactone

Hydrogenation was performed in a Parr hydrogenation apparatus at room temperature: 10 mg of alantolactone were dissolved in 15 mL ethyl acetate, 10 mg of Pd on activated Charcoal (10% Pd, Fluka) were added, and the mixture was shaken for 18 h under a pressure of 50 psi (=3.5 bar). After filtration over Celite the solvent was removed and the residue was purified by column chromatography on silica gel using hexane and a mixture of hexane:ethyl acetate (50:1; 25:1; 10:1; 1:1) as mobile phases. The obtained fractions were monitored by TLC (silica gel $F_{254}$, 0.25 mm (Merck); solvent mixtures: hexane and hexane:ethyl acetate (10:1); spray reagents: vanillin (1%) and $H_2SO_4$ (10%) in EtOH) and HPLC [stationary phase: Zorbax Rx-C18 column (4.6 mm×25 cm), particle size: 5 μm (Rockland Technology); gradient system: 30–85% MeCN in 20 min; UV detection: 205 nm). The main fraction (6 mg) consisted of 3,5,8a-trimethyl-3a,5,6,7,8,8a,9,9a-octahydro-3H-naphtho[2,3-b]furan-2-one and of 3,5,8a-trimethyl-decahydro-3H-naphtho[2,3-b]furan-2-one (=tetrahydroalanto-lactone) in a ratio of 45:55. The constitution was established by 1D and 2D NMR experiments and by MS. The NMR data of both compounds were identical with those published earlier.[19,20]

### Cell culture

RAW 264.7 cells obtained from the American Type Culture Collection (ATCC, TIB 71, Maryland, USA) were cultured in Dulbecco's Modified Essential Medium with 4 mM L-glutamine and 4.5 g/l glucose (DMEM, endotoxin level <0.005 EU/mL, Bio Whittaker, Bioproducts, Heidelberg, Germany), supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL Life Technologies, Eggenstein, Germany). Cells were maintained at 37 °C, 5% $CO_2$ and used for experiments between passages 5 and 20. In general, confluent cells were stimulated with 1 μg/mL LPS (*E. coli*, Serotype 055:B5 Sigma, Deisenhofen, Germany). Sesquiterpene lactones were dissolved in 10% dimethyl sulfoxide (DMSO) at a concentration of 10 mM and further diluted in phosphate-buffered saline (PBS). Final concentrations of DMSO in cell culture supernatants never exceeded 0.1%, which was shown not to interfere with the used assays. Pyrrolidinedithiocarbamate (50 μM; PDTC; Calbiochem, Bad Soden, Germany), a known inhibitor of NF-κB activation and iNOS induction, was used as positive control.

### Nitrite assay (Griess assay)

Cells were seeded in 96-well plates ($8\times10^4$/200 μL/well), cultured for two days, and then incubated with or without lipopolysaccharide (LPS) in the absence or presence of the test compounds for 20 h. As a parameter of NO synthesis nitrite concentration was assessed in the supernatant of RAW 264.7 macrophages by the Griess reaction.[21] Briefly, 100 μL of cell culture supernatant was removed and combined with (i) 90 μL 1% sulfanilamide in 5% $H_3PO_4$ and (ii) 90 μL 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in $H_2O$ in a 96-well plate, followed by spectrophotometic measurement at 550 nm (reference wavelength 620 nm) using a SPECTRA microplate reader (SLT-Labinstruments). Nitrite concentrations in the supernatants were determined by comparison with a sodium nitrite standard curve. Experiments were performed at least three times and performed in triplicate.

### Cell viability (MTT assay)

Cell respiration, an indicator of cell viability, was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan.[22] After removal of supernatants for nitrite determination cells were incubated at 37 °C with MTT (0.5 mg/mL) for 45 min The medium was aspirated, and cells were solubilized in DMSO (250 μL) for at least 3 h in the dark. The extent of reduction of MTT was quantified by OD measurement (550 nm).

### NF-κB binding activity (electrophoretic mobility shift assay)

Cells were seeded in 6-well plates ($4\times10^5$/mL), cultured for two days, and then incubated with or without lipopolysaccharide (LPS) in the absence or presence of the test compounds. Test compounds were pre-incubated 1

h before LPS stimulation. In order to measure NF-κB binding activity cells were activated with LPS for 1 h. Experiments set up in parallel to measure nitrite accumulation were performed using an incubation time of 20 h.

Nuclear extracts were prepared as described previously.[23] Briefly, cells were washed with PBS, suspended in 400 μL ice cold hypotonic buffer A (10 mM HEPES; pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DDT; 0.5 mM PMSF), and kept on ice for 15 min; 25 μL Nonidet NP-40 (10%) was added, the tubes immediately vortexed for 10 s, and the homogenate centrifuged (30 s, 12,000×*g*). The pellet was suspended in 50 μL hypertonic buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) and vigorously rocked for 15 min (4 °C). The extract was centrifuged (5 min, 12,000×*g*) and the supernatant (nuclear protein) immediately frozen at −70 °C. Protein concentration was determined by the Lowry method.[24] Nuclear protein was used for electrophoretic mobility shift assay (EMSA) as described previously.[25] Briefly, a double-stranded oligonucleotide containing the most common NF-κB consensus sequence (22mer, Promega, Mannheim, Germany) was 5′-end-labeled with [γ-$^{32}$P]-ATP (3000 Ci/mmol; Amersham, Braunschweig, Germany) using the T4 polynucleotide kinase (Promega, Mannheim, Germany). Binding reactions were performed incubating 50,000–200,000 cpm radio-labeled oligonucleotide with nuclear protein extracts (10 μg protein) at a final reaction volume of 15 μL (10 mM Tris–HCl, pH 7.5; 1 mM $MgCl_2$; 50 mM NaCl; 0.5 mM DTT; 4% glycerol, 0.5 mM EDTA; 2 μg poly dI-dC (Promega, Mannheim, Germany)) for 30 min at room temperature. Nucleoprotein–oligonucleotide complexes were separated by electrophoresis on a 4.5% non-denaturing polyacrylamide gel (100 V) and the gel exposed to an X-ray film overnight (−70 °C, intensifying screen). Specificity of the DNA–protein complex was confirmed by competition with a 100-fold excess of unlabeled NF-κB (competitor) and AP-2 (non-competitor) oligonucleotides (Promega, Mannheim, Germany), respectively. At least three independent experiments were performed. Quantification was performed by densitometry (EASY plus system, Herolab, Wiesloch, Germany).

### Statistical analysis

Nitrite determination and cell viability were performed in triplicate. All experiments including gel-shift assays were repeated at least two times. Results are expressed as mean value±SEM. Statistical comparisons were made by ANOVA followed by a Dunnett multiple comparisons test. $P$ values $<0.05$ were considered significant.

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