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NF-κB Inhibitors from *Eurycoma longifolia*

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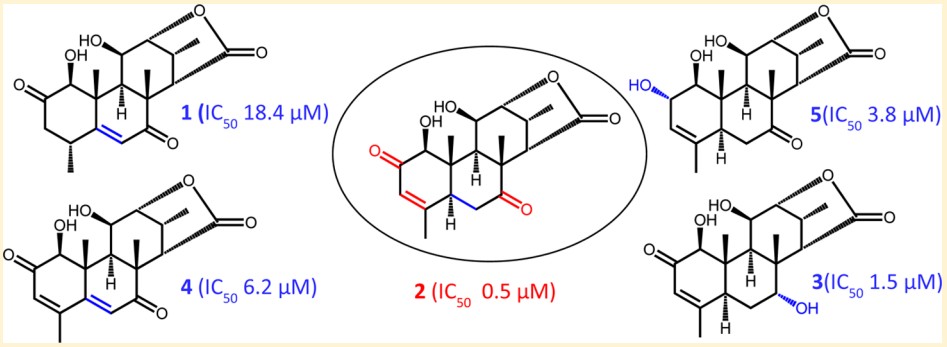
Supporting Information



ABSTRACT: The roots of *Eurycoma longifolia* have been used in many countries of Southeast Asia to alleviate various diseases including malaria, dysentery, sexual insufficiency, and rheumatism. Although numerous studies have reported the pharmacological properties of *E. longifolia*, the mode of action of the anti-inflammatory activity has not been elucidated. Bioguided isolation of NF-κB inhibitors using an NF-κB-driven luciferase reporter gene assay led to the identification of a new quassinoid, eurycomalide C (**1**), together with 27 known compounds including 11 quassinoids (**2**−**12**), six alkaloids (**13**−**18**), two coumarins (**19**, **20**), a squalene derivative (**21**), a triterpenoid (**22**), and six phenolic compounds (**23**−**28**) from the extract of *E. longifolia*. Evaluation of the biological activity revealed that C_{19}-type and C_{20}-type quassinoids, β-carboline, and canthin-6-one alkaloids are potent NF-κB inhibitors, with IC_{50} values in the low micromolar range, while C_{18}-type quassinoids, phenolic compounds, coumarins, the squalene derivative, and the triterpenoid turned out to be inactive when tested at a concentration of 30 μM. Eurycomalactone (**2**), 14,15β-dihydroklaieanone (**7**), and 13,21-dehydroeurycomanone (**10**) were identified as potent NF-κB inhibitors with IC_{50} values of less than 1 μM.

Eurycoma longifolia Jack. (Simaroubaceae) is a shrub or tree distributed in countries of Southeast Asia. It is known locally as "cay ba binh" in Vietnam, "pasak bumi" in Indonesia, and "tongkat ali" in Malaysia. The roots of this plant are used in traditional medicine to alleviate various diseases, such as malaria, dysentery, glandular swelling, and sexual insufficiency.[1] In Vietnam, besides the common usages, a decoction and an alcoholic extract of the roots of *E. longifolia* are used for the treatment of rheumatism.[2] Several compounds such as quassinoids, canthin-6-one alkaloids, β-carboline alkaloids, squalene derivatives, tirucallane-type triterpenes, and biphenyl-neolignans were reported as major components, which possess antimalarial, antiulcer, and antiplasmodial properties and aphrodisiac activities.[3−12]

The anti-inflammatory action of *E. longifolia* has not been investigated, except for a recent study, which reports that this plant has stabilizing properties on human red blood cell membranes.[13] The transcription factor NF-κB is a key regulator of many pro-inflammatory pathways, and therefore its inhibition results in anti-inflammatory effects.[14] In order to investigate a potential NF-κB inhibition, HEK-293/NF-κB-luc cells were used, which is a stable cell line containing an NF-κB-driven luciferase reporter gene that was successfully applied previously for activity profiling of a variety of medicinal plant extracts.[15−18] The methanol extract of the roots of *E. longifolia* revealed promising NF-κB inhibitory effects (66.9 ± 3.2%) at a concentration of 10 μg/mL. Therefore, a bioguided isolation procedure was conducted to identify the active principle(s), which led to the isolation of 28 compounds including a new quassinoid (**1**). The NF-κB inhibitory activities of isolates were determined in a cell-based model, and determinations of their IC_{50} values were performed for the most active of these.

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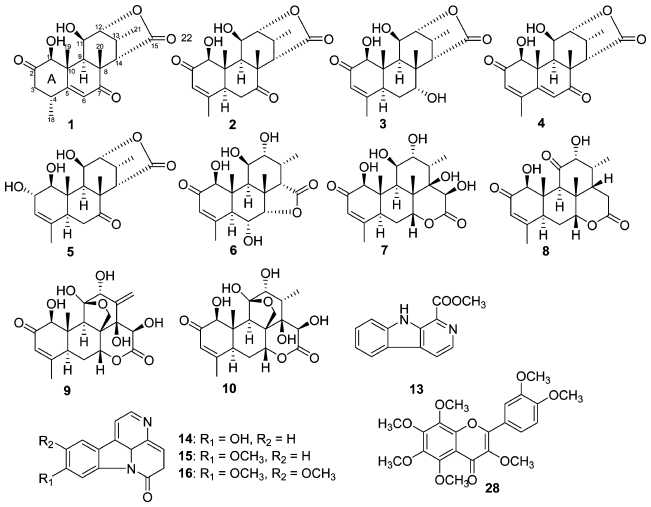
RESULTS AND DISCUSSION

The methanolic root extract of *E. longifolia* was separated by liquid−liquid extraction with water and solvents of increasing polarity (*n*-hexane, diethyl ether, ethyl acetate, and *n*-butanol). The fractions obtained were dried, redissolved in DMSO, and assayed for their ability to inhibit the TNF-α-induced activation of NF-κB. HEK-293/NF-κB-luc cells were treated with test materials at a concentration of 10 μg/mL (positive control: parthenolide 5 μM) and stimulated with TNF-α. Cell viability was determined simultaneously by CellTracker Green CMFDA staining.[15] The diethyl ether and ethyl acetate fractions showed a significant NF-κB inhibitory activity and no significant cytotoxicity (see Figure S1, Supporting Information). Thus, these fractions were separated by Sephadex LH-20 and silica gel column chromatography as well as fast centrifugal partition chromatography to give a new quassinoid, eurycomalide C (**1**), and 27 known compounds (**2**−**28**).

Compound **1** was obtained as colorless plates. The HRESIMS spectrum displayed a quasimolecular ion at *m*/*z* 347.1478 ([M − H]$^-$), consistent with the chemical formula $C_{19}H_{24}O_6$. The IR (1759 cm^{-1}, 1686 cm^{-1}) and UV (234 nm, log ε 3.91) spectra suggested the presence of an α,β-unsaturated ketone of a C_{19}-type quassinoid. The ^{1}H NMR spectrum of **1** showed signals due to an olefinic proton (δ_H 5.90), three oxymethines (δ_H, 4.79, 4.36, 4.08), four methines (δ_H 2.98, 2.92, 2.82, 2.23), a methylene (δ_H 2.72, 2.37), two tertiary methyl groups (δ_H 1.44, 1.38), and two secondary methyl groups (δ_H 1.26, 1.18). The ^{13}C NMR spectrum of **1** revealed 19 signals including those for two carbonyl groups (δ_C 206.9, 198.6), a pair of olefinic carbons (δ_C 165.5, 122.7), a γ-lactone carbonyl carbon (δ_C 176.4), and three oxygen-substituted carbons (δ_C 81.4, 83.4, 69.3). These data closely resembled those of eurycomalactone (**2**), except for the higher field shift of the signal of the olefinic protons (**1**: δ_H 5.90; **2**: δ_H: 6.10), the methylene protons (**1**: δ_H 2.72, 2.37; **2**: δ_H 2.81, 2.76), and the additional secondary methyl groups present. Accordingly, **1** should have a $\Delta^{5,6}$ moiety instead of the $\Delta^{3,4}$ unit of eurycomalactone (**2**). This is consistent with HMBC correlations observed between the olefinic proton at δ_H 5.90 with C-10 (δ_C 49.4) and C-4 (δ_C 34.2) as well as between the methylene proton at δ_H 2.72 and C-2 (δ_C 206.9), C-4 (δ_C 34.2), and C-5 (δ_C 165.5). Therefore, the double bond was located unambiguously at $\Delta^{5,6}$ conjugated with the ketone at C-7. The axial (β) orientation of H-4 was deduced from coupling constants between H-3 and H-4 ($J_{3a,4}$ = 13.5 and $J_{3e,4}$ = 3.9) and could be verified by a ROESY experiment revealing cross-peaks between the protons of the C-19 methyl group and H-4 as well as between the protons of the C-18 methyl group and H-3e (2.37), which showed a further correlation with H-1. Thus, the structure of eurycomalide C was proposed as **1**, representing, to the best of our knowledge, a new quassinoid.

The known constituents were identified as eurycomalactone (**2**),[19] 7α-hydroxyeurycomalactone (**3**),[20] 5,6-dehydroeurycomalactone (**4**),[4] eurycolactone E (**5**),[21] longilactone (**6**),[22] 14,15β-dihydroklaieanone (**7**),[23] 11-dehydroklaieanone (**8**),[24] eurycomanone (**9**),[22,25,26] 13,21-dehydroeurycomanone (**10**),[22] laurycolactone A (**11**),[5] laurycolactone B (**12**),[5] 1-methoxycarbonyl-β-carboline (**13**),[27] 9-hydroxycanthin-6-one (**14**),[28] 9-methoxycanthin-6-one (**15**),[28] 9,10-dimethoxycanthin-6-one (**16**),[9] 5-methoxy-4-hydroxycanthin-6-one (**17**),[29] canthin-6-one 9-*O*-β-D-glucoside (**18**),[30] scopoletin (**19**),[31] fraxidin (**20**),[31] eurylene (**21**),[12] pedunculoside (**22**),[32] vanillic acid (**23**), vanillic aldehyde (**24**), syringic acid (**25**), 1,1′-biphenyl-3,3′-dicarboxylic acid (**26**), isoaloeresin D (**27**),[33] and 3,5,6,7,8,3′,4′-heptamethoxyflavone (**28**).[34] Compound identification was carried out by means of mass spectrometry and NMR spectroscopy as well as by comparison of physical and spectroscopic data with those of reference compounds reported in the literature.

Among these compounds, quassinoids and alkaloids were found as the main components in the active fractions. It is interesting that a chromone derivative (isoaleoresin D, **27**), which so far has been reported only in the genus *Aloe*, was identified also as a constituent of *E. longifolia*. In addition, a triterpenoid (pedunculoside, **22**) and a flavonoid (3,5,6,7,8,3′,4′-heptamethoxyflavone, **28**) were isolated for the first time from this plant.



All isolated compounds (**1**−**28**) were tested for their ability to inhibit the NF-κB pathway in TNF-α-stimulated HEK-293/NF-κB-luc cells at an initial concentration of 30 μM. C_{19}-type (**1**−**6**) and C_{20}-type quassinoids (**7**−**10**), alkaloids (**13**−**16**), and the flavonoid **28** exhibited more than 50% inhibition (Table S1, Supporting Information). These compounds were further analyzed to determine their corresponding IC_{50} values (Table 1). Compounds **2**, **7**, and **10** turned out to be potent NF-κB inhibitors with IC_{50} values less than 1 μM (0.5, 1.0, and 0.7 μM, respectively). Compounds **3**−**6**, **8**, **9**, **14**, and **15** exhibited IC_{50} values ranging from 1.5 to 7.4 μM. Less active were compounds **1** and **16**, showing IC_{50} values of 18.4 and 19.5 μM, respectively, and compounds **13** and **28**, with IC_{50} values higher than 20 μM. The order of their activities was **2** > **10** > **7** > **3** > **8** > **9** > **5** > **14** > **6** > **4** > **15** > **1** > **16** > **28** > **13**. C_{18}-type quassinoids (**11** and **12**), coumarins (**19** and **20**), phenolic compounds (**23**−**27**), the squalene derivative (**21**), and the triterpenoid (**22**) did not show discernible inhibitory effects against NF-κB. Thus, C_{19}-type and C_{20}-type quassinoids and alkaloids may be considered as the major anti-inflammatory principles of *E. longifolia*. Among the quassinoids, eurycomalactone (**2**) showed the most potent NF-κB inhibitory activity, whereas 9-hydrocanthin-6-one (**14**) was the most potent inhibitor of the alkaloids isolated.

Quassinoids are known for cytotoxicity[35] against some cell lines (CaOv-3, HeLa, HepG2, HM3KO, MCF-7), while others (MDBK, Vero) seem to be not affected. Accordingly, the isolated quassinoids were investigated for their effects on cell viability. At a concentration of 30 μM, none of the tested

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Table 1. IC_{50} Values and the Corresponding CI_{95} Values of the NF-κB Inhibition in TNF-α-Stimulated HEK-293/NF-κB-luc Cells of the Test Compounds

compound	IC_{50} (μM)	CI_{95} (μM)
eurycomalide C (**1**)	18.4	16.9−20.1
eurycomalactone (**2**)	0.5	0.3−0.7
7α-hydroxyeurycomalactone (**3**)	1.5	1.3−1.6
5,6-dehydroeurycomalactone (**4**)	6.2	5.3−7.4
eurycolactone E (**5**)	3.8	3.2−4.5
longilactone (**6**)	4.7	3.7−5.9
14,15β-dihydroxyklaieanone (**7**)	1.0	0.8−1.2
11-dehydroklaieanone (**8**)	1.9	1.8−2.1
eurycomanone (**9**)	2.4	2.0−2.9
13,21-dehydroeurycomanone (**10**)	0.7	0.6−0.9
1-methoxycarbonyl-β-carboline (**13**)	29.3	20.3−42.4
9-hydroxycanthin-6-one (**14**)	3.8	3.3−4.4
9-methoxycanthin-6-one (**15**)	7.4	6.6−8.2
9,10-dimethoxycanthin-6-one (**16**)[a]	19.5	13.0−29.4
3,5,6,7,8,3′,4′-heptamethoxyflavone (**28**)	23.3	18.4−29.4
parthenolide (positive control)	1.5	1.3−1.8

[a]Tested as the corresponding HCl salt, dissolved in water.

compounds (Table S1, Supporting Information) showed growth inhibitory activity against HEK-293/NF-κB-luc cells. This is in line with recent studies that investigated the acute toxicity of a diethyl ether fraction of *E. longifolia* and some of its constituents in a mouse model. After oral application, the LD_{50} value of the diethyl ether fraction was 2.31 g/kg body weight, while one of the isolated quassinoids, eurycomanone (**9**), showed an LD_{50} value of 122.5 μM/kg (0.05 g/kg) body weight.[36] The same study evaluated also effects in a brine shrimp toxicity assay, affording LD_{50} values of 144.8, 323.5, 3.5, and 10.3 μg/mL for compounds **6**, **7**, **9**, and **10**, respectively. Interestingly, the acute toxicity-guided fractionation afforded only quassinoids of the C_{20}-type (**7**−**10**), while other types [the C_{18}-type (**11** and **12**), the C_{19}-type (**1**−**6**)] were not detected. A recent clinical study using a standardized water-soluble extract of *E. longifolia* (Physta) containing 0.8−1.5% eurycomanone (**9**) (200 mg twice a day) did not reveal adverse effects.[37] From this it can be concluded that discrepancies in cytotoxicity data of quassinoids are likely due to the different cell model used and varying assay conditions. However, under the particular experimental conditions used in the present study the isolated compounds had no cytotoxic effects.

Among the isolated alkaloids, 1-methoxycarbonyl-β-carboline (**13**) exhibited weak NF-κB effects, with an IC_{50} value of 29.3 μM. In a previous study, another β-carboline alkaloid (4,8-dimethoxy-1-vinyl-β-carboline) isolated from *Melia azedarach* L. var. *japonica* was reported to suppress NO synthesis in LPS/interferon γ-activated RAW 264.7 cells through the inhibition of iNOS protein expression due to decreased mRNA transcription.[37] Moreover, the mechanism of this effect was explained by the capability of β-carboline alkaloids to suppress the NF-κB signaling pathway through inhibition of IKK activity in LPS-stimulated RAW 264.7 macrophages.[38] Although the content of compound **13** in *E. longifolia* roots is only low (based on the isolated amount), it might contribute to the inhibitory NF-κB effect of the extracts.

Among the five isolated canthin-6-one alkaloids, 5-methoxy-4-hydroxycanthin-6-one (**17**) and canthin-6-one-9-*O*-β-D-glucoside (**18**) were inactive at a concentration of 30 μM, while 9-hydroxycanthin-6-one (**14**), 9-methoxycanthin-6-one (**15**), and 9,10-dimethoxycanthin-6-one (**16**) exhibited good NF-κB inhibitory effects. In terms of preliminary structure−activity relationship observations, the hydroxy group at position C-9 seems to be more important than a methoxy group (**14**, IC_{50} 3.8 μM; **15**, IC_{50} 7.4 μM). In addition, methylation of the second phenolic OH group at position C-10 results in a further reduction of the NF-κB inhibitory effect (**16**, IC_{50} 19.5 μM). Canthin-6-one alkaloids have been reported as cytotoxic and antimalarial constituents of *E. longifolia*;[7] however, to the best of our knowledge, this is the first time canthin-6-one alkaloids were found to be NF-κB inhibitors.

The main constituents of the active extracts of *E. longifolia* were identified as quassinoids belonging to the C_{18}-type (**11** and **12**), C_{19}-type (**1**−**6**), and C_{20}-type (**7**−**10**). It is interesting that C_{18}-type quassinoids did not show inhibitory activity, while C_{19}- and C_{20}-type quassinoids were found to be responsible for the effect of the extracts that exhibited NF-κB inhibition in the low μM range (IC_{50} values from 0.5 to 18.4 μM). Preliminary structure−activity relationship data could be established for this compound class. First, since the cyclopentenone in ring A of C_{18}-type quassinoids abrogated this effect, the six-membered ring A is clearly necessary for such activity. Second, the double bond between carbons 3 and 4 seems to be more relevant than the double bond between carbons 5 and 6 (compound **1** gave a higher IC_{50} value (>30 times) than compound **2**). Third, exchange of one of the hydroxy groups at C-2 or C-7 by a carbonyl functionality resulted in a significant decrease of NF-κB inhibition (compound **2**, IC_{50} 0.5 μM; compound **3**, IC_{50} = 1.5 μM; compound **5**, IC_{50} = 3.8 μM).

In conclusion, the present results demonstrated that the methanolic extract of the roots of *E. longifolia* has potent NF-κB inhibitory effects. Investigation of the active fractions led to identification of basically two main compound classes responsible for the activity: (i) C_{19}- and C_{20}-type quassinoids and (ii) β-carboline and canthin-6-one alkaloids. The observed bioactivity of the isolated compounds might be a first indication for their molecular mode of action and will help to elucidate the traditional use of the root of *E. longifolia* against inflammation. The study resulted in the discovery of canthin-6-one alkaloids as a new compound class acting as NF-κB inhibitors.

EXPERIMENTAL SECTION

General Experimental Procedures. The melting point was recorded on a DSC 7 apparatus (Perkin-Elmer, Norwalk, CT, USA). The optical rotations were determined with a Perkin-Elmer 341 polarimeter (Wellesley, MA, USA) at 20 °C. The ultraviolet (UV) spectra were recorded on a Shimadzu UV-1800 spectrophotometer. FTIR spectra were recorded on a Bruker IFS 25 FTIR spectrometer connected to an IR microscope (Bruker Optics, Ettlingen, Germany) in transmission mode "4000−600 cm^{-1}", using ZnSe disks of 2 mm thickness. 1D- and 2D-NMR experiments were recorded on a Bruker DRX 300 (Bruker Biospin Rheinstetten, Germany) or Bruker Advance II 600 NMR spectrometer; NMR solvents were MeOH-d_4/$CDCl_3$/DMSO-d_6/pyridine-d_6 with 0.03% TMS (Eurisotop Gif-Sur-Yvette, France) used as internal standard. HRESIMS were measured on a Bruker mikrOTOF-QII mass spectrometer. LC analyses were carried out using an HP 1100 system (Agilent, Waldbronn, Germany) equipped with autosampler, DAD, and column thermostat. Separations were performed on an Agilent Zorbax SB-C18 80A (150 × 4.6 mm i.d., 3.5 μm) column and a Merck (VWR, Darmstadt, Germany) LiChroCART 4-4 guard column with LiChrospher 100 RP18 (5 μm) packing. A mobile phase consisting of 0.02% TFA in H_2O (v/v) (solvent A) and MeOH (solvent B) was employed with gradient elution (0 min, 90:10 (A:B); 50 min, 20:80; 51 min, 2:98; 60 min,

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2:98). The detection wavelength was 254 nm, and the thermostat was set at 35 °C. The injection volume was 10 μL; the flow rate was 0.3 mL/min. For LC-ESIMS experiments, the HPLC system was coupled to a Bruker (Bruker Daltonics, Bremen, Germany) Esquire 3000plus ion trap, replacing solvent A with a solution of 0.9% formic acid and 0.1% acetic acid in H_2O (v/v). The MS parameters were as follows: ESI positive mode; spray voltage −4.5 kV; nebulizer gas 35 psi; drying gas flow rate 8.00 L/min; *m/z* range 100−1500. In this work, a fast centrifugal partition chromatography (FCPC) (Kromaton, France) apparatus, equipped with Gilson 302/803C pump system model 302 (Villiers-la-Bel, France), was used. Column chromatography was performed using Sephadex LH-20 (Pharmacia Biotech AB, Stockholm, Sweden) and silica gel 60 (0.040−0.063 mm; Merck, VWR, Darmstadt, Germany) as stationary phases. TLC was carried out on silica gel 60 F254 plates (VWR, Darmstadt, Germany). All solvents used for isolation were purchased from VWR International (Darmstadt, Germany). Solvents for HPLC were obtained from Merck (Darmstadt, Germany). Ultrapure water was produced by a Sartorius Arium 611 UV water purification system (Göttingen, Germany). Parthenolide and human recombinant TNF-α were purchased from Sigma-Aldrich, Vienna, Austria.

Plant Material. Roots of *E. longifolia* were collected in Chua Chan-Mountain (Dong Nai/Vietnam) in September 2010 and identified by Prof. Tran Hung (Department of Pharmacognosy/Faculty of Pharmacy, University of Medicine and Pharmacy of Ho Chi Minh City). A voucher specimen (DN107) is stored at the Department of Pharmacognosy/Faculty of Pharmacy, University of Medicine and Pharmacy of Ho Chi Minh City, Vietnam.

Extraction and Isolation. Extraction was carried out with 4 kg of the milled and air-dried roots, which were percolated with 60 L of MeOH at room temperature. The obtained solution was evaporated to dryness using a rotavapor at 35 °C, yielding 320.6 g of crude extract. The initial separation was performed by means of liquid−liquid extraction; 310.4 g of crude extract was suspended in 1.0 L of water and extracted with *n*-hexane (500 mL × 6), diethyl ether (500 mL × 6), and ethyl acetate (500 mL × 10) followed by *n*-butanol (500 mL × 6). Each of combined organic layers as well as the aqueous layer was evaporated to dryness, affording *n*-hexane (Eury-Hx, 13.0 g), diethyl ether (Eury-DE, 6.2 g), ethyl acetate (Eury-Et, 21.8 g), *n*-butanol (Eury-Bu; 38.5 g), and water (Eury-Wa; 160.2 g) fractions. The diethyl ether and ethyl acetate fractions obtained, which showed the most promising anti-inflammatory effects, were separated by chromatographic means to isolate the pure compounds.

The Eury-DE fraction (5.5 g) was subjected to Sephadex LH-20 CC, eluted with MeOH to obtain 11 fractions (EuryA1 to EuryA11). Fraction EuryA9 was applied to silica gel CC (DCM/MeOH, 99.5:0.5 to 98.0:2.0, v/v) to afford compound **15** (4.6 mg). Fraction EuryA9-12 (29.8 mg) was rechromatographed by silica gel CC (EtOAc/MeOH/H_2O, 8:1.8:0.2, v/v/v) to afford compound **14** (12.0 mg). Fraction EuryA8 was separated by Sephadex LH-20 CC, eluted with CH_2Cl_2/acetone (85:15, v/v) to afford 19 fractions. Compound **15** (8.0 mg) was isolated from fraction EuryA8-6 by silica gel CC. Subfraction EuryA8-18 was purified by Sephadex LH-20 CC with MeOH as eluent to yield compound **26** (16.9 mg). A crystalline precipitate of a methanol solution of EuryA7 was separated by filtration and purified by silica gel CC to afford compounds **19** (21.5 mg) and **17** (24.0 mg). The noncrystalline residue of fraction EuryA7 was applied to FCPC (*n*-hexane/$CHCl_3$/MeOH/H_2O, 1:3:3:2, lower phase: mobile phase) to afford 10 fractions (EuryA7-R1 to EuryA7-R10). Fraction EuryA7-R1 was separated by Sephadex LH-20 CC (CH_2Cl_2/acetone, 85:15, v/v) and further purified by silica gel CC (CH_2Cl_2/MeCN, 9:1, v/v) to obtain compound **13** (7.0 mg). Fraction EuryA7-R4 was applied to Sephadex LH-20 CC (CH_2Cl_2/acetone, 85:15, v/v) to afford compounds **20** (12.5 mg) and **24** (15.0 mg). Fraction EuryA7-R7 was separated by Sephadex LH-20 CC (MeOH), yielding compound **23** (13.5 mg). Compound **25** (6.9 mg) was isolated from fraction EuryA7-R6 by Sephadex LH-20 CC (MeOH, followed by MeOH/H_2O, 1:1, v/v). Fraction EuryA7-R10 was further chromatographed on Sephadex LH-20 CC (MeOH, followed by MeOH/H_2O; 1:1, v/v) to yield compound **7** (35.8 mg). Fraction EuryA6 formed crystals in MeOH, which were purified by Sephadex LH-20 CC (CH_2Cl_2/acetone, 85:15, v/v) to obtain compound **2** (75.2 mg). The residue of EuryA6 was applied to Sephadex LH-20 CC (CH_2Cl_2/acetone, 85:15, v/v) to afford 20 fractions. Fraction EuryA6-R6 was subjected to FCPC (*n*-heptane/EtOAc/MeOH/H_2O, 1:1:3:1, upper phase: mobile phase), then purified by Sephadex LH-20 CC (CH_2Cl_2/acetone; 85:15, v/v) and silica gel CC (CH_2Cl_2/MeCN, 95:5, v/v) to give compounds **3** (13.9 mg), **4** (11.7 mg), and **1** (12.0 mg). Fraction EuryA6-R7 was separated by silica gel CC (CH_2Cl_2/MeCN, 95:5, v/v) to obtain compound **12** (20.49 mg) and compound **11** (13.34 mg). Fraction EuryA2 was purified by Sephadex LH-20 CC (MeOH); recrystallization of subfraction EuryA2-2 in $CHCl_3$ afforded compound **21** (80 mg).

Eury-Et (10.5 g) was subjected to Sephadex LH-20 CC, eluted with MeOH, to yield 10 fractions (EuryB1 to EuryB10). Fraction EuryB8 was subjected to silica gel CC (CH_2Cl_2/MeOH; gradient 100:0 to 98:2, v/v) to obtain compound **16** (27.4 mg). Fraction EuryB7 was separated by FCPC (*n*-hexane/CH_2Cl_2/MeOH/H_2O, 0.5:3:3:2, lower phase: mobile phase) and then purified by Sephadex LH-20 CC (MeOH) to afford compound **7** (153.1 mg) and compound **18** (6.8 mg). Fraction EuryB6 was applied to silica gel CC (CH_2Cl_2/MeOH, gradient 10:0 to 8.8:1.2, v/v) to yield 20 fractions. Fraction EuryB6-2 was subjected to Sephadex LH-20 CC (CH_2Cl_2/acetone, 85:15, v/v) and silica gel CC (CH_2Cl_2/MeOH, 9:1, v/v) to obtain compound **28** (5.2 mg) and compound **8** (9.3 mg). Fraction EuryB6-6 was recrystallized from CH_2Cl_2/MeOH (8:2) to yield compound **5** (9.7 mg). Compounds **6** (39.52) and **10** (25.15 mg) were obtained from fractions EuryB6-8 and EuryB6-11 by Sephadex LH-20 CC (MeOH/H_2O, 1:1, v/v), respectively. Fraction EuryB5 was subjected to silica gel CC (CH_2Cl_2/MeOH/H_2O; gradient 10:1:0.5 to 10:4:3, v/v/v), yielding 16 subfractions. Fraction EuryB5-9 and EuryB5-13 were purified by Sephadex LH-20 CC (MeOH/H_2O; 1:1, v/v) to furnish compounds **9** (15.0 mg) and **27** (27.3 mg), respectively. Fraction EuryB5-15 was also subjected to Sephadex LH-20 CC (MeOH/H_2O; 1:1, v/v) and was further purified by silica gel CC to obtain compound **22** (4.5 mg).

*Eurycomalide C (**1**):* colorless plates ($CHCl_3$); mp 243−245 °C; $[\alpha]_D^{20}$ 0 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 234 (3.91) nm; IR (film) ν_{max} 3494, 3356, 2918, 2850, 1759, 1686 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.90 (1H, s, H-6), 4.79 (1H, td, *J* = 4.9, 10.0 Hz, H-11), 4.36 (1H, brd, *J* = 4.7 Hz, H-12), δ 4.08 (1H, brs, H-1), 2.98 (1H, s, H-14), 2.92 (1H, m, H-13), 2.82 (1H, m, H-4), 2.72 (1H, dd, *J* = 3.9, 13.1 Hz, H-3a), 2.37 (1H, t, *J* = 13.5 Hz, H-3b), 2.23 (1H, d, *J* = 3.8 Hz, H-9), 1.44 (3H, s, H-20), 1.38 (3H, s, H-19), 1.26 (3H, d, *J* = 6.1 Hz, H-18), 1.18 (3H, d, *J* = 7.0 Hz, H-21); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 206.9 (C, C-2), 198.6 (C, C-7), 176.4 (C, C-15), 165.5 (C, C-5), 122.7 (CH, C-6), 83.4 (CH, C-12), 81.4 (CH, C-1), 69.3 (CH, C-11), 52.2 (CH, C-14), 49.4 (C, C-10), 48.1 (C, C-8) 47.6 (CH, C-9), 34.2 (CH, C-4), 31.7 (CH, C-13), 22.7 (CH_3, C-20), 18.2 (CH_3, C-19), 18.2 (CH_3, C-18), 16.5 (CH_3, C-21); ESIMS (positive) *m/z* 349.0 $[M + H]^+$, 719.1 $[2M + Na]^+$; HRESIMS *m/z* 347.1478 $[M - H]^-$ (calcd for $C_{19}H_{23}O_6$, 347.1500).

Transformation of Compound 16 into the Corresponding HCl Salt. For pharmacological investigations, all isolated compounds were dissolved in DMSO in a suitable concentration. Since compound **16** showed a low solubility in DMSO, the compound was converted into the corresponding hydrochloride in order to obtain a better solubility; therefore, 2 mg of compound **16** was dissolved in 1 mL of dichloromethane and extracted two times with 1.0 mL of 0.1 N HCl. The aqueous layer was evaporated to dryness to obtain the corresponding salt of compound **16**.

NF-κB Activity and Cell Viability. HEK293/NF-κB-luc cells (Panomics, RC0014) were used for the determination of NF-κB activity and cell viability, as previously described.[15] The cells were cultured at 37 °C and 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) with 100 μg/mL hygromycin B, 100 U/mL benzylpenicillin, 100 μg/mL streptomycin, 2 mM glutamine, and 10% fetal bovine serum (FBS). The cells were stained for 1 h in serum-free medium supplemented with 2 μM CellTracker Green CMFDA (C2925; Invitrogen). Since this

fluorescent probe is retained inside living cells, it can be used to monitor cell membrane integrity and has been widely used to quantify viable cells.[15,39] Afterward, cells were reseeded in 96-well plates (4×10^4 cells/well) in phenol red-free and FBS-free DMEM overnight. Cells were then pretreated with the investigated samples or with solvent vehicle (0.1% DMSO in culture medium) for 30 min and stimulated with TNF-α (2 ng/mL) for 4 h. Then, cells were lysed in a luciferase lysis buffer (Promega; E1531), and the luminescence of the firefly luciferase and the fluorescence of the CellTracker Green CMFDA were quantified with a Genios Pro plate reader (Tecan, Grödig, Austria). For quantification of NF-κB activity, the luciferase-derived signal from the NF-κB reporter was normalized by the CellTracker Green CMFDA-derived fluorescence to account for differences in cell number. Potential differences in cell viability were detected by comparison of the CellTracker Green CMFDA fluorescence of the solvent vehicle treated cells and cells treated with the indicated samples.

Statistical Analyses. Nonlinear regression (with sigmoidal dose response) was used to calculate the IC_{50} values using GraphPad Prism 4.03 (GraphPad Software, Inc.). Statistical differences were compared with ANOVA analysis followed by Tukeys' test. A p-value < 0.01 was considered significant.

■ ASSOCIATED CONTENT

Supporting Information

Effect of the *E. longifolia* extract, chromatographic fractions, and isolated compounds at a concentration of 30 μM, including the cell viability data, structures of additional known compounds, and 1D- and 2D-NMR spectra of eurycomalide C (**1**) are available free of charge via Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

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