Introduction

The transcription factor NF-κB plays a key role in the control of genes involved in the innate and adaptive immune response. Its aberrant regulation is implicated in the pathogenesis of several disease states, including inflammation and autoimmune disorders [1]. NF-κB signaling is involved in cancer development and progression [2]. Furthermore, it is responsible for certain kinds of chemoresistance [3]. In consistence with its role as a central mediator in inflammatory responses, NF-κB is implicated in the aging process [4] and the development of various metabolic diseases [5], with beneficial effects observed upon its inhibition [6]. Therefore, the NF-κB signaling pathway appears as a promising drug...
target [7], which is also susceptible to the bioactivity of numerous small molecules from plant origin [8–11]. The roots of Bupleurum chinense DC (Apiaceae) have a long history in traditional medicine and have been used in China for the treatment of inflammatory disorders and infectious diseases [12, 13]. The main constituents of this plant are triterpenoid sapogenins ("saikosaponins"), which were reported to have in vitro and in vivo anti-inflammatory, immunomodulatory [14], and hepatoprotective [15] activities. One of the major constituents, saikosaponin D, and its epimer, saikosaponin A, were reported to inhibit NF-κB activation [16].

However, there are no further studies that address the NF-κB inhibitory activity of additional saikosaponin analogues from this plant. We report here the isolation of 12 saikosaponins/saikogenins, of which nine have been tested for their potential to inhibit NF-κB transactivation in a cell-based in vitro luciferase reporter gene assay.

Results and Discussion

The dichloromethane (DCM) extract of B. chinense roots inhibited NF-κB-driven gene expression in a cell-based in vitro luciferase reporter assay at a concentration of 10 µg/mL by 83%. The MeOH soluble part of the DCM extract was separated by column chromatography (CC) on MCI® gel. Aliquots of the collected fractions were combined based on the TLC profiles into 10 pooled fractions that were tested for NF-κB inhibition. Activity was detected in pooled fractions 5–8, with the highest activity in pooled fraction 6 (91% inhibition, 10 µg/mL), and a moderate activity in pooled fractions 5 (47% inhibition at 10 µg/mL) and 7 (52% inhibition at 10 µg/mL) (Fig. 15, Supporting Information). From MCI fractions belonging to the pooled fractions 4, 5 and 6, 18 compounds were isolated (▶ Fig. 1), including one sapogenin (1), saponins (2–12), polyines (13–17), and a phospholipid (18). Their structures were determined by 1D and 2D NMR (COSY, HSQC, HMBC) and mass spectrometry (HR-ESI-MS), and by comparison of the spectral data with literature values. Data regarding the inhibition of the NF-κB transactivation activity by pooled fractions 1–10 are shown in Fig. 15 of the supporting information. Also spectroscopic and spectrometric data for the identification of known compounds (1–3, 5, 7–14, and 17–18) are provided in the supporting information.

Compound 6 was obtained as a white amorphous powder. Its HR-ESI-MS displayed a quasi-molecular ion peak at 779.4567 [M + H]+, which, together with 13C NMR data provided a molecular formula of C42H66O13. The 1H NMR spectrum showed the presence of a cis-olefinic group, two anomic protons, and six tertiary methyl groups. Through interpretation of the 2D NMR data, all proton and carbon resonances were assigned. HMBC correlation was observed between one of the olefinic protons (δ 5.94, H-11) and a tertiary carbon (δ 85.6, C-13), which in turn showed HMBC correlation with a methylene group (δ 76.3, CH2-28), suggesting an ether linkage between them. This indicated a triterpenoid aglycone similar to that of saikosaponins A (8) or D (7) [17]. The NMR resonances belonging to the rings A and B of compound 6 were almost superimposable to that of compounds 7 and 8. The major difference was the existence in compound 6 of a ketone group (δ 214.8, C-16) instead of a hydroxylated methine. This was supported by the HMBC correlations of this ketone carbon with H-15, H-22, and H-28, as well as lower field shifts for C-14 (ΔδC = 6.6 Hz), C-15 (ΔδC = -9.9 Hz), C-17 (ΔδC = 11.3 Hz), and C-18 (ΔδC = 4.2 Hz). Therefore, the aglycone part of compound 6 was determined as 13,28-epoxy-3β,23-dihydroxy-olean-11-en-16-one [18]. The remaining 12 carbon signals, together with two anomic protons at δ 4.53 (d, J = 7.7 Hz) and 4.38 (d, J = 7.7 Hz), suggested the existence of two sugar units. Further analysis of the resonance data revealed a β-glucopyranosyl and a β-fucopyranosyl. As only D-configuration of both sugars have been reported in saikosaponins, we tentatively assigned the D-configuration based on biosynthetic considerations. HMBC correlations between signals at δ 4.53 (H-1′) and δ 85.2 (C-3′) suggested the connection of β-glucopyranosyl to C-3′ of the β-fucopyranosyl moiety. The connection of the sugar chain to C-3 of the aglycone was deduced from the HMBC correlation between signals at δ 4.38 (H-1′) and δ 83.0 (C-3). The sugar sequence was supported by fragment ions at m/z 617 [M+H-Glc]+, 471 [M+H-Glc-Fuc]+, and 453 [M+H-Glc-Fuc-H2O]+. Therefore, the structure of compound 6 is established as 13,28-epoxy-23-hydroxy-olean-11-en-16-one-3β-yl O-β-D-glucopyranosyl(1→3)-β-D-fucopyranoside. Com-
and 13C NMR data. The UV spectrum showed strong absorption maxima at 245 (sh), 251, and 260 (sh) nm, indicating the existence of an heteroannular diene [19]. This was further supported by the 1H NMR signals of cis-olefinic protons at δ 6.45 and 5.58, and 13C NMR signals at δ 137.2, 132.7, 127.1, and 126.7. Through interpretation of the 2D NMR data, all proton and carbon resonances were assigned. The13C NMR data of the aglycone part were nearly superimposable to that of saikosaponin B2 ([17]), except for the upper field shift for C-4 (ΔδC 3.7 ppm), and down-field shifts for C-3 (ΔδC 7.2 ppm), C-5 (ΔδC 8.5 ppm), C-24 (ΔδC 3.7 ppm), and C-23 (ΔδC 36.9 ppm), which revealed the absence of a 23-hydroxyl group in compound 4 [17]. At the same time, upper field shifts were observed in the 1H NMR for H-3 (ΔδH 0.46 ppm) and H-5 (ΔδH 0.42 ppm), owing to the releasing of steric hindrance from 23-hydroxyl group. When compared with saikosaponin E (9), these atoms, together with other atoms belonging to the ring A of the pentacyclic skeleton of compound 4, shared nearly identical chemical shifts with their counterpart in compound 9 [17]. Therefore, the aglycone of compound 4 was determined to be 16-epi-saikogenin C [19]. The remaining carbon signals, together with two anomic protons at δ 4.55 (d, J = 7.7 Hz) and 4.32 (d, J = 7.7 Hz), could be readily assigned to the sugar moiety. As in the case of compound 6, through further analysis of the NMR data, the existence of a β-glucopyranosyl and a β-fucopyranosyl was determined, and the configuration of both of them was also tentatively assigned as D for biogenetic reasons. Their sequence was elucidated by HMBC correlations at δ 4.55 (H-1)′/δ 84.9 (C-3′) and δ 3.10 (H-3′)/δ 105.6 (C-1′″), which suggest the connection of β-glucopyranosyl to C-3′ of the β-fucopyranosyl moiety. HMBC correlations at δ 3.62 (H-3)/δ 105.6 (C-1′) and δ 4.32 (H-1)/δ 83.1 (C-3) supported the connection of the sugar chain to C-3 of the aglycone. This linking pattern was supported by mass fragment peaks at m/z 747 [M+H-H2O]⁺, 585 [M+H-H2O-Glc]⁺, and 439 [M+H-H2O-Glc-Fuc]⁺. Therefore, the structure of compound 4 is established as 16α,28-dihydroxyolean-11,13(18)-diene-3β-yl O-β-D-glucopyranosyl-(1 → 3)-β-D-fucopyranoside. To our knowledge, it is reported here for the first time and is given the trivial name saikosaponin W.

The remaining triterpenoids were identified by comparison of their spectral data with literature values as saikogenin D ([17]), prosaikogenin D ([20]), saikosaponins B3 ([17]), B1 (5) [17], D (7) [17], A (8) [17], E (9) [17], B4 (10) [17], B3 (11) [17], and T (12) [21].

Nine of these compounds (1, 2, 3, 4, 6, 8, 10, 11, and 12) present in bioactive pooled fractions 5 and 6 were tested at 30 µM for their NF-κB inhibitory activity in vitro (Fig. 2a). At 30 µM, compound 6 markedly reduced fluorescence of the vital dye (cell tracker green [CTG]), indicating cytotoxicity and therefore a false positive result of NF-κB inhibition in the cell-based luciferase assay. Compound 8 (saikosaponin A) showed a strong decrease of NF-κB-dependent luciferase gene expression in vitro. However, it also showed a significant reduction of CTG fluorescence at 30 µM, indicating cytotoxicity of this compound. Compounds 10–12 showed no NF-κB inhibition and did not show any cytotoxicity. Interestingly, compounds 1–4 showed NF-κB inhibitory activities, but no or low cytotoxicity (Fig. 2). For compounds 1 and 4, which show strong NF-κB inhibition at 30 µM, IC₅₀ values were determined. Compound 1 possesses an IC₅₀ val-
ue of 14.0 µM and compound 4 an IC₅₀ value of 14.4 µM in the NF-κB-dependent luciferase reporter gene assay in vitro (Fig. 3).

Some preliminary structure-activity relationships can be deduced regarding the aglycone moiety. Both of the highly cytotoxic saponins (6 and 8, type “b” and “c” Fig. 1) possess an ether bridge between C-13 and C-28 of the aglycone part. The three NF-κB inactive saponins (10–12, type “d” Fig. 1), which are artifacts from the isolation process [22], have a methoxy group at C-11 in common. The NF-κB active and less cytotoxic compounds (1–4, type “a” Fig. 1) possess a heteroannular 11,13 (18)-diene system [22].

Compounds 1–3 showed a mild decrease in CTG fluorescence, indicating low cytotoxicity at 30 µM (without reaching significance for 1). Their activities in terms of inhibiting NF-κB transactivation seem to correlate inversely with the number of sugars attached at C-3 of the aglycone. The aglycone saikogenin D (1) was the strongest inhibitor (91% inhibition) with no significant cytotoxicity. Compound 2, identified as prosaikogenin D, with one sugar (fucose) attached, was less active (75% inhibition). Compound 3, identified as salkosaponin B2, and possessing one more sugar (glucose), was even less active (20% inhibition). Compound 4 (salkosaponin W), the C-23-deoxyanalogue of compound 3 (salkosaponin B2), was one of the most active, decreasing NF-κB activation by 76.9 ± 1.4%, with no indication of cytotoxicity at 30 µM. These data suggest, in the case of type “a” saponin/triterpenoid, that NF-κB inhibition is favorably influenced by a shorter (or no) sugar chain at aglycone C-3 position, and no C-23 hydroxyl group.

Compound 8, which is one of the two major saponins of Radix Bupleuri, can be transformed into its prosapogenin and genuine sapogenin after oral administration. Its incubation with intestinal flora led also mainly to genuine sapogenin. But when incubated in gastric juice, type “a” saponins turned out to be the major products [23]. Actually, Chinese pharmacopoeia includes vinegar processing (being fried in vinegar) of Radix Bupleuri. Due to the heated acidic condition, this process can lead to a sharp increase of the content of type “a” and concomitant decrease of type “c” saponins [24]. The NF-κB inhibition observed for compounds 1, 2, and 4 (type “a”) lend support to this processing method.

Besides one saponin (6), fractions belonging to pooled fraction 6 also contained a small amount of compound 18, obtained as a colorless gum, whose strong affinity to both normal and reversed stationary phases indicated amphiphilic properties. Its 1D and 2D NMR spectra suggested a 1,3-substituted glycerol, an esterified linoleic acid, and a choline moiety [25]. The remaining phosphate unit was identified by positive ESI-MS/MS, which showed a quasi-molecular ion (m/z 520) and a specific fragment at m/z 184 characteristic of a protonated phosphorylcholine head group [26]. Therefore, compound 18 was identified as a lysophosphatidylcholine (lyso-PC) [25]. This is the first time that a lysophosphatidylcholine is isolated from the genus Bupleurum. Compound 18 showed moderate NF-κB inhibition (44%) at 30 µM.

Finally, four C15 polycyctenes (13–16), and one C17 polycyctene (17) were isolated in very small amounts. Their structures were determined through interpretation of NMR and MS spectra as well as by comparison with reference compounds. This is the first time polycyctenes were isolated from B. chinense.

Compounds 13–16 were isolated as amorphous solids. Compound 13 was identified as saikodyne A [27]. Present ¹³C NMR experiments showed a different shift for C-4 than previously reported. The corrected value is outlined in the supporting information. Compound 14 was identified as a reduced derivative of saikodyne B [27]. Compound 17 was identified as falcarnidol, a C17 polycyctene [28].

Compounds 15 and 16 are both reported for the first time. Compound 15 was obtained as a white amorphous solid. The HR-ESI-MS, ¹³C NMR, and HSQC data indicated a molecular formula of C₁₅H₂₂O₂. As in the case of compound 13 and 14, ¹³C NMR spectrum of 15 suggested the presence of four acetylene carbon signals, at δ 84.2, 75.3, 66.2, and 64.1 ppm. Comparison of ¹³C NMR spectra of 15 with those of 13 and 14 suggested the existence of a five-membered aliphatic chain, whose connection to the alkene C-10 was supported by the HMBC correlations at δ 4.08 (H-10)/δ 37.9 (C-11), δ 1.48 (H-11)/δ 72.6 (C-10), δ 1.48 (H-11)/δ 150.7 (C-9). The missing of one of the two pairs of alkene protons in 15 at lower field coincided with the appearing of two vicinal methylene signals at δ 2.42, δ 1.73 (CH₂-2 and CH₂-3). At the same time, up-field shifts were observed for their vicinal hydroxymethylene protons (HO-CH₂). Taking the solvent effect into consideration, the NMR data of 15 are highly superimposable to that of virol C, except for the signals of the longer aliphatic chain in virol C [29,30]. By comparing their optical rotation, the configuration of 15 was determined as 10S [29]. Therefore, the structure of compound 15 was elucidated as (10S,8E)-pentadeca-8-en-4,6-diyne-1,10-diol. We assigned the trivial name saikodyne E to compound 15.

Compound 16 was obtained as a white amorphous solid. The HR-ESI-MS, ¹³C NMR, and HSQC data indicated a molecular formula of C₁₅H₂₆O₂. The ¹³C NMR spectrum of 16 suggested the presence of four acetylene carbon signals, at δ 85.1, 79.4, 75.1, and 69.0 ppm. Like compounds 13 and 14, compound 16 possessed two olefinic bonds. One of the major differences revealed by the HSQC spectrum was the presence of a lower field hydroxymethylene proton (δ 5.19, d, J = 8.3 Hz, H-8) in 16, while in both 13 and 14, a higher field hydroxymethylene proton (δ 4.21, p, J = 5.5 Hz, H-10) was observed. The splitting pattern of the hydroxymethylene proton at δ 5.19, together with the HMBC correlations at δ 5.47 (H-9)/δ 28.5 (C-11), δ 2.13 (H-11)/δ 134.1 (C-10), δ 2.13 (H-11)/δ 129.8 (C-9) revealed the shifting of the double bond from Δ⁸ in 13 and

Liu X et al. Bupleurum chinense Roots: ... Planta Med 2017; 83: 1242–1250

1245
14 to δ3 in 16. The NMR data of 16 are highly superimposable to that of another C17 analogue, (2Z,8S,9Z)-heptadeca-2,9-dien-4,6-diyne-1,8-diol [31]. By comparison of their optical rotations, the configuration of 16 was determined as 8S [31]. Therefore, the structure of compound 16 was elucidated as (2Z,8S,9Z-penta-deca-2,9-dien-4,6-diyne-1,8-diol. Compound 16 was named saikodyne F.

Due to their limited amounts, compounds 13–17 have not been tested for their bioactivity.

In summary, this study examined nine triterpenoids (1–4, 6, 8, and 10–12) for their potential to inhibit NF-κB transactivation activity in vitro in a cell-based NF-κB-driven luciferase reporter gene model in HEK293 cells. Six of these compounds showed NF-κB inhibition. However, four of them appeared also to be cytotoxic, especially the type "b" and "c" saponins 6 and 8. Compound 4, a new saponin belonging to type "a" presented the best NF-κB inhibitory activity in relation to its cytotoxicity. Several polyacetylenes, including two new congeners, have been isolated in trace amounts for the first time from B. chinense. These findings enhance the understanding of this plant as an anti-inflammatory herbal medicine.

Materials and Methods

General experimental procedures

All solvents were obtained from VWR Chemicals and Carl Roth. TLC was performed on Silica gel 60F254 and Silica gel 60 RP-18F254, plates (Merck): detection with a mixture of 1% vanillin and 10% sulfuric acid in dehydrated ethanol. Open CC was carried out with MCI CH-P 20P resin (Mitsubishi Chemical), RP-18 silica gel (25–40 µm, Fuji silica), Sephadex LH-20 (GE Healthcare), and silica gel (15–40 µm, Merck) as stationary phases. Semi-preparative HPLC experiments were performed with L-6200A intelligent pump (at a flow rate of 3 ml/min) and L-4500 DAD detector (Merck-Hitachi) equipped with LicroCART 10 × 250 mm column and L-4500 DAD detector (Merck). For large-scale isolation, 900 g of Material-II was pulverized and subsequently percolated with DCM (10 L). After evaporation to dryness (<40 °C), the DCM extract (36 g) was then partitioned twice between n-hexane and 90% MeOH (1.5:1, v/v), yielding 24.7 g of a defatted, MeOH soluble part, which was subsequently fractionated by CC (Ø: 5.5 cm, L: 55 cm), using MCI CHP-20P resin as the stationary phase and an MeOH-water gradient (40% to 100%, v/v) as the mobile phase, to afford 101 fractions (Fr. 1 to Fr. 101, 700 mL each). After the fractions had been evaporated to dryness, they were transferred into small vials, and reconstituted to 10 mL. For bioassay, 30 µL were taken from each vial, and combined according to their TLC profiles to form 10 pooled fractions, tested for NF-κB inhibition.

MCI fractions belonging to the NF-κB inhibitory pooled fractions 5 and 6 were subjected to Sephadex LH-20 (35% MeOH in water) chromatography, in order to separate polyacetylenes from dominant saponins. The saponin containing subfractions were then subjected to RP-18 (Ø: 4 cm, L: 45 cm; eluted with 60–70% MeOH water solution, v/v) and silica gel (Ø: 3 cm, L: 30–50 cm; eluted with n-hexane-ethylacetate, 3:1 or 2:2:1, v/v) open CC, and finally semi-preparative RP-18 HPLC (35%, 40%, 45%, or 50% MeCN) to afford a sapogenin and eleven saponins, respectively: 1 (2.4 mg) [17], 2 (2.5 mg) [20], 3 (1.5 mg) [17], 4 (2.4 mg), 5 (0.6 mg) [17], 6 (1.3 mg) [18], 7 (5.7 mg) [17], 8 (4.7 mg) [17], 9 (4.4 mg) [17], 10 (20.7 mg) [17], 11 (6.2 mg) [17], and 12 (8.5 mg) [21]. Fractions belonging to pooled fraction 4, which also contained polyacetylenes, were processed in the same way. The polyacetylene enriched subfractions were purified directly by semi-preparative HPLC (isocratic 50% or 69% MeCN) to afford five polyacetylenes, respectively: 13 (0.8 mg) [27], 14 (0.8 mg) [27], 15 (0.9 mg), 16 (0.5 mg), and 17 (0.9 mg) [32]. The isolation of 18 (2.4 mg) was achieved through RP-18 SPE (80% MeOH) and semi-preparative RP-18 HPLC (60% MeCN) [25]. Among them, saponins 1–5 and 7–12 were isolated from the combined fractions 37, 38, 39, and 40 (460 mg) belonging to the fifth pooled fraction; polyacetylenes 13–15 were isolated from the combined fractions 33, 34, 35, and 36 (490 mg) belonging to the fourth and fifth pooled fractions; polyacetylene 16 was isolated from the combined fractions 31 and 32 (663 mg) belonging to the fourth pooled fraction; polyacetylene 17 was isolated from the combined fractions 41, 42, 43, and 44 (370 mg) belonging to the fifth pooled fraction; saponin 6 and lysophosphatidylcholine 18 were isolated from the combined fractions 47 and 48 (210 mg) belonging to the sixth pooled fraction. According to HPLC profiles and NMR spectra, all compounds tested for bioactivity are at least 95% purity.

16α,28-dihydroxyoleane-11,13(18)-diene-3β,20-diy-0-β-D-glucopyranosyl-(1→3)-3β-D-fucopyranoside (4, saikosaponin W): white amorphous powder; [α]D20 = 29.0 (c 0.03, MeOH); UV (MeCN/H2O) λmax;
Table 1 $^1$H and $^{13}$C NMR spectroscopic data (in CD$_3$OD) of compounds 4 and 6, $j$ in Hz.

| $\delta^1$H | $\delta^1$C | $\delta^1$H | $\delta^1$C |
|------------|------------|------------|------------|
| 1          | 1.88 (1H)  | 1.02 (1H)  | 1.94 (1H)  | 0.94 (1H)  |
| 2          | 1.95 (1H)  | 1.75 (1H)  | 1.96 (1H)  | 1.80 (1H, $d, j = 14.2$) |
| 3          | 3.18 (1H, $dd, j = 11.7, 4.5$) | 90.6 | 3.63 (H-3) | 83.0 |
| 4          | 4          | 39.4       | 40.3       | 44.1       |
| 5          | 0.85 (1H)  | 56.7       | 1.21 (1H)  | 48.0       |
| 6          | 1.64 (1H)  | 1.46 (1H)  | 19.4       | 1.54 (2H)  | 18.1 |
| 7          | 1.38 (2H)  | 33.4       | 1.50 (1H)  | 1.14 (1H)  | 32.0 |
| 8          | 4.01 (1H, $t, j = 3.2$) | 69.1       | 43.0       |
| 9          | 1.99 (1H, br s) | 54.8 | 1.91 (1H) | 53.8 |
| 10         | 37.5       | 37.1       | 4          |
| 11         | 5.58 (1H, $dd, j = 10.7, 1.2$) | 127.1 | 6.04 (1H, $d, j = 10.4$) | 134.6 |
| 12         | 6.45 (1H, $dd, j = 10.8, 2.9$) | 126.7 | 5.53 (1H, $dd, j = 10.3, 3.1$) | 129.6 |
| 13         | 137.2      | 85.6       | 14         |
| 14         | 42.3       | 50.9       | 15         |
| 15         | 1.93 (1H)  | 32.0       | 2.79 (1H, $d, j = 14.5$) | 1.80 (1H, $d, j = 14.2$) | 45.4 |
| 16         | 4.03 (1H, $t, j = 3.2$) | 69.1 | 214.8 |
| 17         | 45.4       | 57.4       | 18         |
| 18         | 132.7      | 56.3       | 19         |
| 19         | 2.48 (1H, $d, j = 14.7$) | 1.69 (1H, $d, j = 14.8$) | 39.4 | 1.55 (1H) | 1.43 (1H) | 40.0 |
| 20         | 33.2       | 32.4       | 21         |
| 21         | 1.56 (1H)  | 35.8       | 1.51 (1H)  | 1.24 (1H)  | 36.6 |
| 22         | 2.01 (1H)  | 24.5       | 2.11 (1H)  | 1.26 (1H)  | 25.1 |
| 23         | 1.06 (3H, s) | 28.2 | 3.67 (1H) | 3.29 (1H) | 64.7 |
| 24         | 0.84 (3H, s) | 16.5 | 0.71 (3H, s) | 12.7 |
| 25         | 0.92 (3H, s) | 18.6 | 0.98 (3H, s) | 18.8 |
| 26         | 0.73 (3H, s) | 17.5 | 1.21 (3H, s) | 20.1 |
| 27         | 1.23 (3H, s) | 22.1 | 1.02 (3H, s) | 20.7 |
| 28         | 3.74 (1H, $d, j = 11.7$) | 3.26 (1H) | 65.1 | 3.90 (d, $j = 7.9$) | 3.44 (d, $j = 8.0$) | 76.3 |
| 29         | 0.85 (3H, s) | 25.3 | 0.93 (3H, s) | 33.8 |
| 30         | 0.97 (3H, s) | 32.9 | 0.90 (3H, s) | 23.5 |

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| $\delta^1$H | $\delta^1$C | $\delta^1$H | $\delta^1$C |
|------------|------------|------------|------------|
| 1'         | 4.32 (1H, $d, j = 7.7$) | 106.6 | 4.38 (1H, $d, j = 7.7$) | 105.7 |
| 2'         | 3.67 (1H) | 71.9 | 3.65 (1H) | 71.9 |
| 3'         | 3.60 (1H, $dd, j = 9.7, 3.3$) | 84.9 | 3.60 (1H) | 85.2 |
| 4'         | 3.85 (1H) | 72.4 | 3.85 (1H) | 72.3 |
| 5'         | 3.64 (1H) | 71.2 | 3.66 (1H) | 71.3 |
| 6'         | 1.26 (3H, $d, j = 6.4$); | 16.9 | 1.27 (3H, $d, j = 6.4$); | 16.9 |

continued
245 (sh), 251, and 260 (sh) nm; ^1^H and ^1^C NMR (150 MHz, CD3OD) data, see ▶ Table 1; HR-ESI-MS m/z 763.4641 [M – H] (calcd for C_{42}H_{68}O_{12}: 763.4633).

13,28-epoxy-23-hydroxy-olean-11-en-16-one-3 ^β^-O-[^β^-D-gluco-pyranosyl(1 → 3)-[^β^-D-fucopyranoside (6, s a i k o s a p o n i Y): white amorphous powder; ^1^H and ^1^C NMR (150 MHz, CD3OD) data, see ▶ Table 1; HR-ESI-MS m/z 777.4437 [M – H] (calcd 777.4425 for C_{42}H_{65}O_{13}).

(10S,8E)-pentadeca-8-en-4,6-diyne-1,10-diol (15, saikodiyne E): white amorphous solid; [^α]_{D}^20 + 35.1 (c 0.03, MeOH). UV (MeCN/H2O) λ_{max}: 216, 243, 256, 270, 286 nm; ^1^H and ^1^C NMR, see ▶ Table 2; HR-ESI-MS m/z 291.1602 [M + CH3COO] (calcd 291.1596 for C_{17}H_{23}O_{4}).

NF-κB transactivation activity
NF-κB activity was evaluated in HEK293/NF-κB-luc cells (HEK293 cells stably transfected with a NF-κB-responsive luciferase reporter gene Panomics, RC0014) as previously described [33, 34]. Cells were loaded with CTG CMFDA (C2925; Invitrogen), a fluorescent probe that is retained inside living cells [11, 35], and 4 × 10^4 cells/well were seeded in 96-well plates. After 24-h incubation, cells

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### Table 1 Continued

|   | C     | δ_H   | δ_C   | C     | δ_H   | δ_C   |
|---|-------|-------|-------|---|-------|-------|
|   |       |       |       | 6  |       |       |
| 1''| 4.55 (1H, d, J = 7.7) | 105.6 | 4.53 (d, J = 7.7) | 105.7 |
| 2''| 3.28 (1H) | 75.4  | 3.29 (1H) | 75.4 |
| 3''| 3.37 (1H) | 77.7  | 3.36 (1H) | 77.7 |
| 4''| 3.32 (1H) | 71.3  | 3.33 (1H) | 71.2 |
| 5''| 3.28 (1H) | 77.9  | 3.28 (1H) | 77.9 |
| 6''| 3.84 (1H) | 62.5  | 3.84 (1H, dd, J = 12.0,2.0) | 62.4 |

* Measured at 600 MHz (for ^1^H) and 150 MHz (for ^1^C).

### Table 2

|   | C     | δ_H   | δ_C   | C     | δ_H   | δ_C   |
|---|-------|-------|-------|---|-------|-------|
| 15|       |       |       | 16|       |       |
| C | δ_H   | δ_C   | C     | δ_H   | δ_C   |
| 1 | 3.63 (2H, t, J = 6.3) | 61.4  | 4.30 (2H, dd, J = 6.4,1.6) | 61.1 |
| 2 | 1.73 (2H, p, J = 6.7) | 32.3  | 6.23 (1H, dt, J = 11.6,4) | 147.8 |
| 3 | 2.42 (2H, t, J = 7.0) | 16.6  | 5.63 (1H, d, J = 11.1) | 109.1 |
| 4 |       | 84.2  |       | 75.1 |
| 5 |       | 66.2  |       | 79.4 |
| 6 |       | 75.3  |       | 69.0 |
| 7 |       | 74.1  |       | 85.1 |
| 8 | 5.71 (1H, d, J = 15.8) | 109.0 | 5.19 (1H, d, J = 8.3) | 59.0 |
| 9 | 6.24 (1H, dd, J = 15.9,5.8) | 150.7 | 5.47 (1H, ddt, J = 10.1,8.3,1.6) | 129.8 |
| 10 | 4.08 (1H, qd, J = 6.2,1.6) | 72.6  | 5.56 (1H, ddd, J = 10.9,7.6,1.2) | 134.1 |
| 11 | 1.49 (2H, q, J = 7.1) | 37.9  | 2.13 (2H, q, J = 7.1) | 28.5 |
| 12 | 1.41 (1H) | 62.4  | 1.41 (2H, p, J = 7.2) | 30.1 |
| 13 | 1.30 (2H) | 32.9  | 1.32 (2H) | 32.5 |
| 14 | 1.33 (2H) | 23.7  | 1.33 (2H) | 23.6 |
| 15 | 0.91 (3H, t, J = 6.9) | 14.4  | 0.91 (3H, t, J = 7.0) | 14.4 |

* Measured at 600 MHz (for ^1^H) and 150 MHz (for ^1^C).
were pretreated as indicated for 30 min and activated with TNF-α (2 ng/mL, Sigma; T-6676) for 4 h. Cells were then lysed with luciferase lysis buffer (Promega; E1531), the luminescence of the firefly luciferase and the fluorescence of stained cells with CTG (also: CMFDA, 5-chloromethylfluorescein diacetate) were quantified with a GeniosPro plate reader (Tecan). The luciferase signal resulting from the NF-κB reporter activation was normalized by the CTG-derived fluorescence to account for potential differences in cell number. The impact of cytotoxicity on cell numbers was evaluated by comparing fluorescence of the cells treated by the solvent vehicle with that of cells treated with the indicated samples. Parthenolide (Sigma-Aldrich; P0667 ≥ 98% HPLC), a known NF-κB inhibitor, was used as positive control (10 µM). Results were normalized to the solvent control DMSO (0.1%).

Statistical analysis and calculation of IC50 values
Statistical analysis and nonlinear regression were performed using Prism software (version 4.03; GraphPad Software Inc.). To calculate the IC50 values, data were curve fitted and non-linear transformed using a sigmoidal dose response with variable slope. Results of bioassay data are expressed as the mean ± standard deviation (SD) of at least three independent experiments performed in quadruplicate. Results of IC50 values are expressed with 95% confidence interval (CI). Dunnett’s test was used for statistical analyses (*p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant).

Supporting information
Data regarding the inhibition of the NF-κB transactivation activity by pooled fractions 1–10 are shown in Fig. 15 of the supporting information. Also spectroscopic and spectrometric data for the identification of known compounds (1–3, 5, 7–14, and 17–18) are provided in the supporting information.

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Conflict of Interest
The authors declare no conflict of interest.

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