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A prenylbisabolane with NF-κB inhibiting properties from Cascarilla (*Croton eluteria*)

Claudio Campagnuolo,[a] Ernesto Fattorusso,[a,*] Francesca Petrucci,[a] Orazio Taglialatela-Scafati,[a] Giovanni Appendino,[b] Nieves Marquez[c] and Eduardo Muñoz[c]

[a]*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli 'Federico II', Via D. Montesano 49, I-80131 Napoli, Italy*
[b]*DISCAFF, Università del Piemonte Orientale, V.le Ferrucci 33, 28100 Novara, Italy*
[c]*Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Avda Menendez Pidal s/n, 14004 Córdoba, Spain*

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Abstract—Investigation of the bark of *Croton eluteria* Bennett for biologically active compounds has led to the isolation of the new prenylbisabolane **3**, whose structure was assessed by spectroscopic methods. The corresponding known enone **4** and the eudesmane sesquiterpene **2** were also obtained. Compound **3** proved active in selectively inhibiting the induction of NF-κB by tumor necrosis factor-α in T cells.

1. Introduction

The bitter bark of the South American tree *Croton eluteria* Bennett (Euphorbiaceae), commonly known as cascarilla, has been widely used in the traditional folk medicine to replace more valuable medicinal barks like *Cinchona* and *Cascara*.[1] Nowadays its major use is in the field of aromatization, where its fragrance and bitter taste have made it a popular flavoring agent for liqueurs.[1] Chemical investigation of cascarilla started in 1845 when its major secondary metabolite, the clerodane diterpenoid cascarillin (**1**), was isolated.[2] This discovery stands as a milestone in the history of natural products chemistry, since cascarillin was the first diterpenoid to be obtained in pure form, and was also one of the first non-alkaloidal bitter substances isolated from a plant source.

Spurred by the relevance of this plant as a flavoring agent and by the bioactivity of secondary metabolites obtained from different *Croton* species (e.g., anticancer,[3] gastroprotective[4]), we have undertaken a systematic investigation of *C. eluteria*. Our efforts resulted in the isolation of a new halimane (pseudoeleuterin B)[5] and a series of twelve new clerodanes (named eluterins A–K and cascarilladione).[5,6] It was also discovered that the acetone extract of cascarilla strongly potentiates the histamine-stimulated gastric acid secretion. This activity was traced back to cascarillin (**1**), the major constituent of the extract,[5] rationalizing the use of cascarilla in preparations aimed at improving digestion.

Cascarilla is a commercially available and abundant source of structurally complex diterpenoids that can be used as core structure for building libraries of analogues to assay for useful bioactivity. The large scale isolation of its major constituent (cascarillin, ca. 0.31% on the dried plant material) was therefore pursued, providing the opportunity to obtain new minor constituents from the side cuts and mother liquors of the isolation protocol. In the event, while optimizing the chromatographic fractionation of the extract to induce the crystallization of cascarillin, three compounds unavailable from the previous studies were obtained. Using spectroscopic techniques, they were identified in the eudesmane sesquiterpene **2** and in the two prenylbisabolane diterpenoids **3** and **4**. Interestingly, while neither the crude acetone extract from cascarilla nor cascarilline showed any activity in assays for NF-κB inhibition, the new

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* Corresponding author. Tel.: +39 081 678503; fax: +39 081 678552; e-mail: fattoru@unina.it

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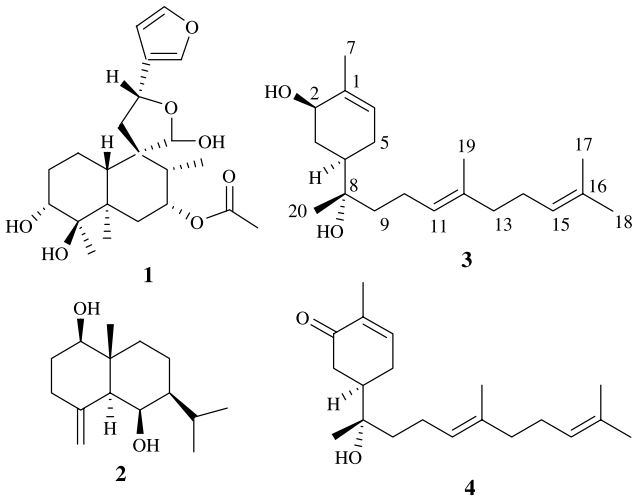

bisabolane **3**, a new natural product, could potently inhibit the induction of this transcription factor mediated by TNFα in a T cell line. The structures of the known compounds **2**[7] and **4**[8] were assigned by comparing their spectroscopic data with those reported in the literature, while that of **3**, obtained as an optically active colorless foam, is detailed here.

2. Results and discussion

Cascarilla bark was powdered, extracted by percolation with acetone at rt and the obtained extract was fractionated by column chromatography with a petroleum ether–EtOAc gradient to give four fractions (A–D). Fraction D was mainly composed by cascarillin, while fractions B and C were separated by sequential application of Sephadex LH-20 column and HPLC on silica gel to give the known eudesmane sesquiterpene **2** (3.8 mg), the novel compound **3** (1.6 mg) and only trace amounts of the known compound **4** (0.4 mg), together with previously described clerodane and halimane diterpenoids.

The electrospray MS (positive ions) of **3** showed a pseudomolecular ion peak at *m/z* 329 $[M+Na]^+$, while the EI spectrum showed the molecular ion peak at *m/z* 306. High resolution measurement of this peak assigned the molecular formula $C_{20}H_{34}O_2$ to **3**. The ^1H and ^{13}C NMR spectra of **3** ($CDCl_3$, Table 1) were analyzed with the help of the 2D HMQC experiment, suggesting the presence of four allylic methyls, an additional methyl singlet at δ_H 1.13, three trisubstituted C=C groups, six sp^3 methylenes, one sp^3 methine, and two oxygen bearing sp^3 carbons [one methine and one unprotonated carbon atom]. Thus, in order to account for the four unsaturation degrees implied by the molecular formula, **3** might be monocyclic. Inspection of the ^1H–^1H COSY spectrum allowed us to deduce the three spin systems evidenced with bold linkages in Figure 1, while analysis of the HMBC spectrum provided information to join the above moieties, thus building up the planar structure of compound **3** (Fig. 1). In particular, $^{2,3}J$ HMBC correlations of the methyl protons at C-7 indicated the presence of a cyclohexene ring that, in turn, was connected to the C_{13} acyclic tail thank to the correlations of H_3-20 with C-4, C-9, and C-8.

Table 1. ^1H and ^{13}C NMR data of **3** in $CDCl_3$

Pos.	δ_H, mult., *J* in Hz	δ_C, mult.
1		136.4, s
2	4.19, m	71.1, d
3a	2.28, dd, 10.5, 4.5	33.4, t
3b	1.36, ddd, 10.5, 7.7, 7.5	
4	1.75, m	42.1, d
5a	2.01[a]	27.1, t
5b	1.89, ddd, 9.5, 9.5, 1.8	
6	5.48, br s	123.7, t
7	1.77, s	18.9, q
8		74.1, s
9	1.53, m	39.7, t
10	2.08[a]	22.3, t
11	5.14, t, 6.3	124.6, d
12		136.3, s
13	1.99, m	39.9, t
14	2.08[a]	26.9, t
15	5.04, t, 6.3	124.4, d
16		131.9, s
17	1.68, s	26.4, q
18	1.60, s	18.2, q
19	1.61, s	16.0, q
20	1.13, s	23.1, q

[a] Overlapped with other signals.

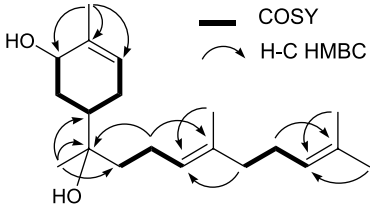

Figure 1. Spin systems deduced through the COSY spectrum and key $^{2,3}J$ H→C correlations exhibited by the HMBC spectrum of compound **3**.

The *E* geometry of $\Delta^{11,12}$ finds evidence in the NOE correlation between H-11 and H_2-13, while the spatial coupling of H-2 with H-4, evidenced through a ROESY experiment, pointed to the *cis*-diaxial orientation of these protons. The coupling constants measured for H-2 and H-4 are in full agreement with their axial orientation. Further experiments aimed at establishing the absolute configurations at C-2, C-4, and C-8 of **3** were not attempted due to the limited amount of material available. However, because of the co-occurrence of compounds **3** and **4** in *C. eluteria*, it is reasonable to assume that C-4 and C-8 in **3** share the same absolute configuration of the corresponding carbons in **4**. Compound **3** is a new natural product, but it was recently reported, without full spectroscopic characterization, as a synthetic intermediate en route to **4**.[9] The measured (+12.5) and the reported (+14.8)[9] $[\alpha]_D$ value for **3** were in good agreement, confirming the absolute configuration of the natural product.

The occurrence of compounds **3** and **4** in *C. eluteria* is remarkable, since prenylbisabolane diterpenoids are very rare, and less than a dozen of members of this class have been isolated from both marine[10] and terrestrial sources.[11] It is interesting to note that the related monocyclic monoterpenoids (*p*-menthane skeleton) and monocyclic sesquiterpenoids (bisabolane skeleton) are, on the contrary, extremely common. Given the large occurrence of diterpenoids in cascarilla, the prenylbisabolane framework of **3** and **4** is seemingly formed by direct cyclization of geranylgeranyl diphosphate. Also the isolation of a sesquiterpene from a plant from the Euphorbiaceae family is worth noting.

As part of a screening aimed at discovering new chemotypes of anticancer and antiinflammatory natural products from plants, the acetone extract of *C. eluteria* and its major component, cascarillin, were evaluated in an assay of NF-κB inhibition.[12] NF-κB is a transcription factor involved in activation of a large number of genes in cancer, inflammation, apoptosis, and other situations requiring rapid reprogramming of gene expression.[13] NF-κB regulates the transcription of proinflammatory cytoquines, chemokines, cytokine receptors, adhesion molecules, and key enzymes in the inflammatory process [cycloxygenase-2 (COX-2) and inducible nitric-oxide synthase (iNOS)] and is a hot target in biomedical research.[14] Remarkably, while no activity was detected in the crude acetone extract and in cascarillin, compound **3** showed to inhibit the induction of NF-κB by tumor necrosis factor-α. In particular, the 5.1 cell line, a clone derived from the leukemia T cell line Jurkat, was used. This is stably transfected with a plasmid containing the luciferase gene driven by HIV-1-LTR promoter, which contains two NF-κB binding sites required for TNFα-induced transactivation.[15] The cells were pre-incubated (30 min) with increasing doses of compound **3**, stimulated with TNFα (5 ng/mL) for 6 h and finally the luciferase activity measured in the cell lysates. In this cellular assay the luciferase activity correlates with the levels of NF-κB activation. Figure 2 shows how compound **3** inhibits, in a dose dependent manner, the TNFα-mediated HIV-1 LTR gene transcription through the NF-κB pathway. To study whether the inhibition was due or not to cytotoxicity, we incubated 5.1 cells with compound **3** (100 μM) for 24 h and the cell cycle analyzed by propidium iodide staining and flow cytometry. We did not found significant differences in the percentage of hypodiploid cells (apoptotic cells) detected in untreated compared to treated cells (Fig. 3). Moreover, other phases of the cell cycle were not affected by compound **3**. These results indicate that, at the concentrations tested, the anti-NF-κB activity of this compound was quite specific and not due to cellular toxicity.

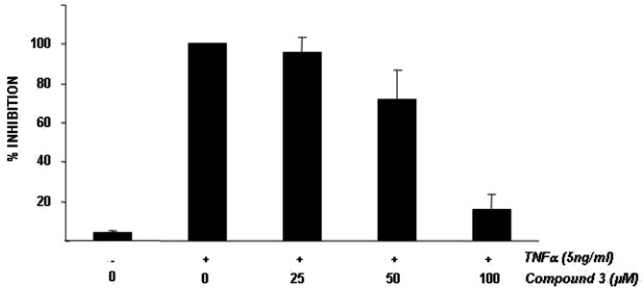

Figure 2. Compound **3** inhibits NF-κB dependent luciferase gene expression. 5.1 cells were pre-treated with different doses of compound **3** and treated with TNFα for 6 h, after which luciferase activity was measured. The results show the percentage of inhibition over the TNFα stimulation. Values are means ± SD of four independent experiments.

3. Conclusions

Other prenylbisabolanes derivatives have been described as insecticide agents,[8] but the present study discloses for the first time anti-NF-κB activity for a member of this rare class of diterpenoids. Many NF-κB inhibitors possess highly electrophilic sites in their molecule,[16] but, interestingly, compound **3** lacks elements of this type, and its pharmacophore could therefore not be predicted.

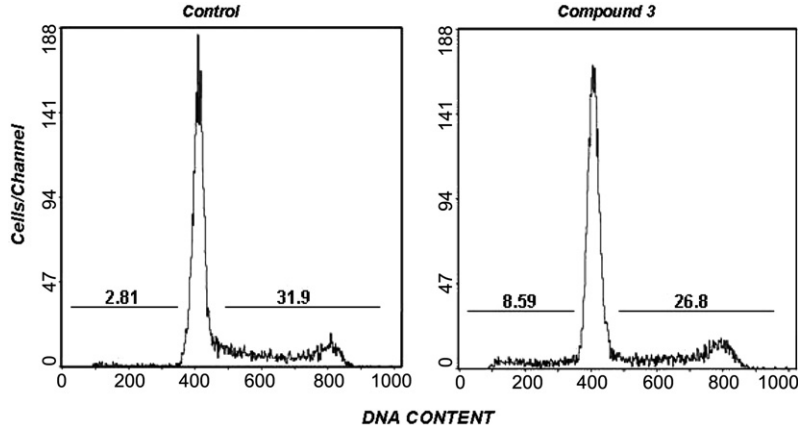

Figure 3. Effects of compound **3** on the cell cycle. 5.1 cells were treated with compound **3** (100 μM) for 24 h and the cell cycle studied by PI staining and flow cytometry. The numbers in the figures represent the percentage of apoptotic cells (left) and the percentage of cells cycling through phases S and G_2/M of the cycle (right). Results are representative of three independent experiments.

Active compounds can be present in crude extracts in concentrations too low to exert a detectable biological response in bioassays, as exemplified by compound **3**. The discovery of this interesting chemotype of NF-κB inhibitor could have been easily missed in a screening program based on the evaluation of crude extracts, since, on account of its very low concentration (ca. 80 ppm), no activity of this type can be detected in unfractionated extracts from cascarilla. This observation, while questioning the value of negative bioassay results obtained on crude extracts, also highlights the relevance of commercially available medicinal and aromatic plants as a chemical library, since the modern chromatographic methods, especially when removal of the major constituents by crystallization is feasible, allow the isolation of compounds present in ppm concentration, magnifying the opportunity to discover novel bioactivity.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured in $CHCl_3$ on a Perkin–Elmer 192 polarimeter equipped with a sodium lamp (λ_{max} = 589 nm) and a 10 cm microcell. IR (KBr) spectra were recorded on a Bruker model IFS-48 spectrophotometer. Low resolution ESI (positive ions) were performed LCQ FINNIGAN MAT mass spectrometer; low and high resolution EI mass spectra (70 eV, direct inlet) were performed on a VG Prospec (FISONS) mass spectrometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Bruker AMX-500 spectrometer; chemical shifts are referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26, δ_C = 77.0). One bond heteronuclear ^1H–^{13}C connectivities were determined with the HMQC experiment. Two and three bond ^1H–^{13}C connectivities were determined by HMBC experiments optimized for a $^{2,3}J$ of 7.5 Hz. Nuclear Overhauser effect (NOE) measurements were performed by 2D ROESY experiments. Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861 apparatus using Merck SI60 (230–400 mesh) stationary phase. High performance liquid chromatography (HPLC) separations in isocratic mode were achieved on a Beckmann apparatus equipped with refractive index detector and with Phenomenex LUNA SI60 (250 × 4 mm) columns.

4.2. Plant material

Cascarilla, *C. eluteria* Bennet, was purchased from Minardi, Bagnacavallo (RA). A voucher specimen is held at DISCAFF.

4.3. Extraction and isolation

Powdered bark of *C. eluteria* (Cascarilla, 250 g) was extracted by percolation with acetone at rt (4 × 2 L). Evaporation of the pooled extracts left a brown gum (19 g). This was fractionated by column chromatography (silica gel) with a petroleum ether–EtOAc gradient (from 9:1 to 1:9) to give four fractions (A–D). Fraction D (petroleum ether–EtOAc 4:6) was mainly composed by cascarillin (1.02 g, 0.31%). Fractions B (1.3 g) and C (1.2 g) (petroleum ether–EtOAc 7:3 and 5:5, respectively) were complex mixtures and could be poorly resolved by column chromatography on silica gel. Thus, they were combined and then chromatographed on Sephadex LH-20 (MeOH–CH_2Cl_2 1:1) affording six sub-fractions (A1–A3 and B1–B3). HPLC purification on silica gel (*n*-hexane–EtOAc 55:45) of fraction B2 (48 mg) afforded the known eudesmane sesquiterpene **2** (3.8 mg), the novel compound **3** (1.6 mg) and the known compound **4** (0.4 mg). Chromatographic separations of other subfractions were achieved as previously detailed[5,6] and led to the isolation of already described clerodane and halimane diterpenoids.

4.4. Compound 3

Colorless amorphous solid. $[\alpha]_D^{25}$ +12.5 (*c* 0.01, $CHCl_3$); IR (KBr): *ν* 3370, 2965, 2918, 1450, 1377 cm^{-1}; CD (CH_3CN): λ_{max} 213 nm ($\Delta\varepsilon$ +8.5). ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra ($CDCl_3$): Table 1. ESIMS (positive ions): *m/z* 329 $[M+Na]^+$. EIMS (70 eV): *m/z* 306 $[M]^+$ (5), 288 (20), 237 (20), 195 (10), 177 (20), 155 (40), 111 (100). HREIMS: *m/z* 306.2604 (calcd for $C_{20}H_{34}O_2$, *m/z* 306.2559).

5. Biological assays

5.1. Cell lines and reagents

The 5.1 clone (obtained from Dr. N. Israël, Institut Pasteur, Paris, France) was maintained in exponential growth in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM Hepes, and the antibiotics penicillin (100 U/mL) and streptomycin (100 μg/mL) (Invitrogen, Barcelona, Spain). All the other reagents were from Sigma–Adrich (Barcelona, Spain).

5.2. Luciferase assays

To determine NF-κB-dependent transcription of the HIV-LTR promoter, 5.1 cells were preincubated for 30 min with compound **3** and stimulated with TNFα (5 ng/mL) for 6 h. Then the cells were lysed in 25 mM Tris–phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity (relative light units, RLU) was measured using an Autolumat LB 9501 (Berthold Technologies, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI), and protein concentration was measured by the Bradford method. Then RLU/μg protein was calculated and the results expressed as the percentage of inhibition were 100% activity was assigned to the transcriptional activity induced by TNFα alone.

5.3. Cell cycle analysis

Treated and control cells were washed in PBS and fixed in ethanol (70% for 24 h at 4 °C). Then, the cells were

washed twice with phosphate-buffered saline (PBS) and subjected to RNA digestion (RNase-A, 50 U/mL) and propidium iodide (20 mg/mL) staining. The cell cycle was analyzed by cytofluorimetry in an EPIC XL flow cytometer (Beckman Coulter, Fullerton, CA). With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining make it possible to determine the percentage of sub-diploid cells (sub-G_0/G_1 fraction or apoptotic cells).

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