A Polyisoprenylated Benzophenone from Cuban Propolis

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A novel polyisoprenylated benzophenone (**1**) has been isolated from an ethanol extract of Cuban propolis. Its structure has been determined using high-field 2D NMR techniques. Compound **1** showed significant antimicrobial and antifungal activity against a variety of bacteria and yeasts.

In previous studies, polyisoprenylated 2,4,6-trihydroxybenzophenones and alkyl aryl ketones, of mixed shikimate and acetate biogenesis, have been isolated from plants belonging to the Guttiferae family, specifically from the genera *Garcinia, Clusia,* and *Rheedia* (subfamily Clusoideae).[1−6] In these benzophenone derivatives the acetate-derived trihydroxy-aromatic ring is usually prenylated. In addition, alkylation of this ring results in the formation of a complex bridged bicyclic[1,2] or tricyclic system.[3−6] Several of these compounds have shown antimicrobial, antifungal, and anti-HIV activity.[4,7] Phytochemical investigations have also revealed benzophenone derivatives in the fruits, roots, leaves and twigs of plants in the Guttiferae. Resins exuded by the flowers of several *Clusia* species and collected by tropical pollinating bees (*Apis mellifera*) during their nest construction[8] are the dominant sources of tropical Venezuelan propolis,[9] and the resins contain polyprenylated benzophenones as major components. Propolis is a complex mixture of beeswax, small amount of sugars, and plant exudates collected by honeybees.[10] The composition of propolis depends on the place and time of collection, and more than 160 constituents have been identified so far, among which phenolic compounds are major constituents.[11] Because of its range of antibiotic, antifungal, antinflammatory, anticancer, and antioxidant activities,[12] there is renewed interest in the composition of propolis. *Clusia* and *Baccharis* spp. are significant sources of tropical Brazilian and Chilean propolis.[13−14] In this investigation, we examined propolis collected in a small area, Nuevitas, in Cuba. We describe the isolation, structural elucidation, and antimicrobial and antifungal activities of a new polyisoprenylated benzophenone.

Compound **1** was obtained from the ethanol extract of propolis by HPLC. Analysis of spectral data suggested that **1** was a polyisoprenylated benzophenone. The IR spectrum showed three bands for carbonyl groups (ν_{max} 1720, 1696, and 1640 cm^{-1}); the lack of any bands at 3550−3250 cm^{-1} indicated the absence of hydroxyl groups.

The molecular formula $C_{33}H_{42}O_4$ of **1** was deduced using MS, ^{13}C NMR, and DEPT analyses. The EIMS of **1** gave a molecular ion at *m/z* 502, identical to that of clusianone,[1,2] and negative FABMS gave the quasimolecular anion peak at *m/z* 501. In the EIMS spectrum, additional peaks at *m/z* 105 and 69 suggested the presence of an unsubstituted benzoyl group and isoprenyl substituents, respectively.

Analysis of the 1D and 2D NMR spectra with homo- and heteronuclear direct or long-range correlations allowed assignment of ^1H and ^{13}C NMR signals, as listed in Table 1. Both the molecular formula and NMR data indicated that **1** was a trioxybenzophenone derivative containing four five-carbon units. The 600 MHz ^1H NMR ($CDCl_3$) spectrum (Table 1) revealed five aromatic proton signals in the region δ 7.28−7.53 attributable to an unsubstituted phenyl group. Two vinyl protons (δ 5.01 and 5.03), suggesting two isopent-2-enyl substituents, four aliphatic methyl proton singlets between δ 1.16 and 1.44, four vinylic methyl groups in the region δ 1.59−1.70, and four allylic protons were observed.

The ^{13}C NMR spectrum indicated an unconjugated (δ 207.8) and two conjugated carbonyls (δ 192.0 and 193.0), a substituted enolic group (δ 169.3), and clearly assignable C-6 (methylene), C-7 (methyne), and C-8 (quaternary) signals comprising part of an enolized bicyclo[3.3.1]nonane-2,4,9-trione ring system, which is a common feature in polyisoprenyl benzophenones.[1−7] Easily identifiable pendant residues included a phenyl ketone (from C-10 to C-16), the *gem*-dimethyl group (C-32 and C-33) correlating by HMBC to each other and to the C-8 quaternary carbon of the main skeleton; a 3-methyl-2-butenyl group (from C-27 to C-31) linked to the methyne at C-7, as evidenced by a COSY experiment, and a second 3-methyl-2-butenyl group (from C-22 to C-26) linked to the basic skeleton at a quaternary carbon. A *gem*-dimethylpyran ring group (C-17 to C-21) was present from signals of two Me on a C bearing an oxygen (δ 1.44 and 1.30 respectively) as a 2,2-dimethylchroman residue.[4] Multiple-bond heteronuclear correlation (HMBC) data unambiguously established the tricyclic core structure of the molecule and that isopentenyl and dimethylpyran groups were substituents at C-5, C-7, and C-3, respectively. Starting from the *gem*-dimethyl group at C-8, correlations were observed between protons of both Me groups (δ_H 1.41 and 1.16) and the quaternary carbon signal C-1 which, from its deshielded position, had to be inserted between three carbonyl groups (C-2, C-9, and C-10). Cross-peaks were also observed between these two methyl groups and the methyne carbon at C-7, and between the C-8 quaternary carbon. Correlations between the C-6 methylene protons and C-8, C-5, and C-4 established that they were respectively in 3- and 2-bond relationships and attached to C-6. The carbon signal at δ 114.2 was assigned to C-3 on the basis of biogenetic considerations and by analogy with known compounds such as clusianone.[1,2] The two 3-methyl-2-butenyl groups were positioned at C-5 and C-7 because allylic methylenes at C-22 (δ 2.46 and 2.57) and at C-27 (δ 1.67 and 2.19) showed diagnostic HMBC correlations to C-4, C-5 and to C-7, C-8, respectively. The

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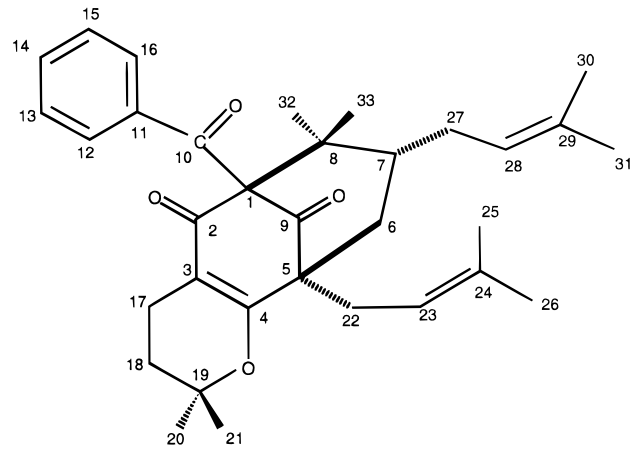
Table 1. ^{1}H and ^{13}C NMR Data of Compound **1** in CD_3OD[a,b]

position	δ_C	DEPT	δ_H (J_{H-H} in Hz)
1	79.9	C	–
2	192.0	C	–
3	114.2	C	–
4	169.3	C	–
5	57.7	C	–
6	40.50	CH_2	1.99 eq dd (13, 3), 1.45 ax dd (13, 10.5)
7	44.1	CH	1.68 m
8	48.3	C	–
9	207.9	C	–
10	193.5	C	–
11	133.4	C	–
12 and 16	127.4	CH	7.53 d (8)
13 and 15	128.4	CH	7.28 t (8)
14	131.5	CH	7.40 t (8)
17	16.5	CH_2	2.40 m, 1.78 m
18	32.0	CH_2	1.81 m, 1.70 m
19	79.1	C	–
20	27.9	Me	1.44 s
21	24.9	Me	1.30 s
22	29.0	CH_2	2.57 dd (14, 8), 2.46 dd (14, 6)
23	119.2	CH	5.03 dd (8, 6)
24	134.0	C	–
25	26.1	Me	1.70 s
26	18.4	Me	1.66 s
27	26.8	CH_2	1.67 ddd (14, 9, 9), 2.19 ddd (14, 7, 1)
28	122.8	CH	5.01 dd (8, 7)
29	133.4	C	–
30	25.8	Me	1.69 s
31	17.4	Me	1.59 s
32	23.7	Me	1.16 s
33	15.9	Me	1.41 s

[a] Chemical shift values are in ppm from TMS, and *J* values (in Hz) are presented in parentheses. Carbon multiplicities were determined using DEPT experiments. All signals were assigned by DQF−COSY, HSQC, and HMBC experiments. [b] Main HMBC correlations are reported in the text.

^{1}H−^{1}H DQF−COSY spectrum provided confirming evidence, showing correlations between H-6 and H-7 and between H-7 and allylic methylene protons at C-27 which, in turn, were correlated to the C-28 vinyl proton. Thus, C-7 is the point of attachment of one of the 3-methyl-2-butenyl groups. Finally the H-17 of the *gem*-dimethyl pyran ring correlated by COSY to H-18, showed HMBC correlations with C-3 and C-4, revealing C-3 as the point of attachment of the *gem*-dimethyl pyran ring. The connectivity pattern inferred by the HMBC spectrum is compatible only with the structure **1**.



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Assignment of relative stereochemistry to **1** was based principally on coupling constant analysis, NOE data obtained from a ROESY spectrum, and on comparison with reference compounds.[7] The basic bicyclic ring system in **1** required that the phenyl ketone on C-1 and the isopentenyl chain on C-5 be equatorial. A 10.5 Hz coupling between the methylene proton H-6ax and H-7 required these protons to be diaxial, thus the isopentenyl group at C-7 was β-equatorial. The H-6eq proton signal showed NOE interaction with H-7β, Me-33β, and one of the methylene protons at C-22. NOE interaction between Me-32α and one of the methylene protons at C-27 and H-6α was also consistent with structure **1**.

From a biogenetic point of view, the structure of **1** suggested a cyclization mechanism of the putative precursors that is different from that which results in the previously reported prenylated benzophenones such as the bicyclic clusianone and tricyclic nemorosonol.[1,2] Compound **1** is the first reported example of a benzophenone derivative in which the *gem*-dimethylpyran group is linked to C-3 and C-4. This process could be either elicited by the bees themselves or by the metabolism of native Cuban Guttiferae species.

Compound **1** was tested for its antimicrobial and fungicidal activities against several Actinomyces, Gram positive and Gram negative bacteria, and yeasts. The results are reported in the Experimental Section. Antibacterial and fungicidal activities are expressed as a MBC (minimum bactericide concentration) and as a MFC (minimal fungicide concentration). Major activity was observed against two Gram positive bacteria (*Streptomyces chartrensis* and *Streptomyces violochromogenes* MBC 50). In the case of Gram negative bacteria and yeasts, MBC and MFC ranged from 600 for *Shigella sonnei* and *Candida albicans* and *Candida tropicalis* to 1200 for *Pseudomonas aeuruginosa* and *Candida parapsilosis*.

Experimental Section

General Experimental Procedures. Melting points were determined using a Bausch & Lomb apparatus. Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. UV spectra were obtained with a Beckman DU 670 spectrophotometer and IR spectra with a Bruker IFS-48 spectophotometer. A Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 for ^{13}C, using the UXNMR software package was used for NMR experiments in CD_3OD. ^{1}H−^{1}H DQF−COSY (double quantum filtered COSY), ^{1}H−^{13}C HSQC, HMBC, and ROESY experiments were obtained using conventional pulse sequences. The EIMS spectrum was obtained from a VG-PROSPEC mass spectrometer (70 eV). The FABMS spectrum was recorded in a glycerol matrix in the negative ion mode on a VG ZAB instrument (Xe atoms of enrgy of 2−6 kV). HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Waters μ-Bondapak C18 column.

Biological Material. The propolis was collected in September 1996. The hives and forage fields are located in a small area, Nuevitas, northern Camagey, Cuba. Dominant species in the forage fields are *Clusia minor* and *Clusia rosea*. A voucher sample (Prop I, 1996) is deposited at the Center for Pharmaceutical Chemistry (CFQ), Havana, Cuba.

Extraction and Isolation. The propolis (2 kg) was extracted with EtOH (3 × 1000 mL) at room temperature and the extract concentrated under reduced pressure. The residue (952 g) was partitioned between hexane and EtOH/H_2O (8:2). The hexane layer was concentrated and partitioned with 5% $NaHCO_3$ solution. The polar phase was neutralized with diluted HCl, extracted with $CHCl_3$ and concentrated. The residue, analyzed by TLC (SiO_2 plates, $CHCl_3$/MeOH 9:1), gave one main spot which was isolated by RP-HPLC (μ-Bondapack

C-18 column, MeOH/H_2O 9:1, flow rate 2 mL/min, $t_r = 13$ min). The product **1** was crystallized from a mixture of EtOH/H_2O to (8:2) yielding 40 mg of pure compound.

Compound 1: white cubes; mp 113.5 °C from EtOH/H_2O, which became yellow in $CHCl_3$ solution on exposure to light; $[\alpha]_D^{25}$ +40° (*c* 0.1, $CHCl_3$); UV (EtOH) λ_{max} 248 and 277 nm; IR (KBr) ν_{max} 3000, 2850, 1720, 1696, 1639, 1603, 1580, 1446, 1383, 1373, 1221, 1151, 1112 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; EIMS (probe) 70 eV, *m*/*z* 502 (23) M^+, 487 (4), 434 (11), 433 (37), 365 (17), 351 (7), 311 (15), 310 (21), 309 (100), 297(9), 295 (15), 105 (42) $(C_7H_5O)^+$, 77 (12) $(C_6H_5)^+$, 69 (15) $(C_5H_9)^+$; negative FABMS *m*/*z* $[M - H]^-$ 501.

Microbiological Screening. The antimicrobial action of propolone A (**1**) against selected bacteria and yeasts was evaluated by the method of Agar plate double radial diffusion,[15] using 6.8×10^8 colony forming units per mL in each case. Susceptibility disks (5-mm diameter) were impregnated with different concentrations of product and then placed on agar plate inoculated with the test bacteria and yeasts. Incubation time was 24 h at 35 °C for bacteria and 48 h at 25 °C for yeasts. After incubation the plates were observed for zone of inhibition. The growth inhibition was judged by comparison with growth in control spots prepared without test compounds. MBC and MFC were the minimal concentration of propolone A (**1**) required to eliminate 99% of the microorganism previously inoculated. Screening was performed against the following microorganisms from American Type Culture Collection (ATCC) collections or from human clinical origin isolated at the Department of Micology of the Calixto Garcia Hospital (*Candida* species), and at the Department of Microbiology, Faculty of Biology of the University of Havana (*Streptomyces* species): Gram type positive (MBC): *S. chartrensis* A-16 (50); *S. violochromogenes* CB-37 (50); *S. aurantiogriseus* D-1 (100); *S. phaeochromogenes* A-22 (100); *S. epidermidis* ATCC 12228 (100); *S. aureus* ATCC 6538 (300). Standard gentamycin and nystatin used as controls gave respectively MBC 10.5 against *S. chartrensis* and MFC 16.2 against *C. albicans* in the same experimental conditions. The results were statistically treated and the confidence intervals were determined at a confidence level $\gamma = 0.95$.

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