# A Hexacyclic *ent*-Trachylobane Diterpenoid Possessing an Oxetane Ring from *Mitrephora glabra*

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**ABSTRACT**
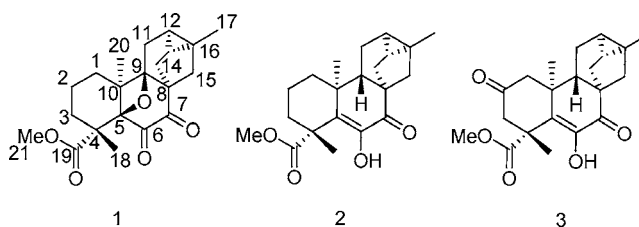


Three new *ent*-trachylobane diterpenoids (1–3) were isolated and structures elucidated from *Mitrephora glabra* Scheff. (Annonaceae). Mitrephorone A (1) possesses a hexacyclic ring system with adjacent ketone moieties and an oxetane ring, both of which are unprecedented among trachylobanes. All compounds were evaluated for cytotoxicity against a panel of cancer cells, where 1 displayed the most potent and broadest activity, and against a battery of antimicrobial assays, where all compounds were approximately equipotent.

As part of our collaborative program to search for new compounds from the plant kingdom with promising anti-cancer activity,[1] *Mitrephora glabra* Scheff. (Annonaceae) was selected for investigation. The genus *Mitrephora*, representing approximately 40 species distributed widely throughout tropical Asia,[2] has been under-explored for biologically active secondary metabolites, and to the best our knowledge, only four species have been studied previously: *M. celebica*,[3,4] *M. maingayi*,[5,6] *M. tomentosa*,[7] and *M. zippeliana*.[8] The

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organic extract of *M. glabra* showed promising anticancer activity in cell cytotoxicity assays, and bioactivity-directed fractionation led to the isolation of a series of three new *ent*-trachylobane diterpenoids (**1**−**3**) and the related known compound *ent*-trachyloban-18-oic acid (**4**).[9,10]

The molecular formula of compound **1** was determined by HRMS to be $C_{21}H_{26}O_5$, establishing an index of unsaturation of nine. Examination of the $^1H$, $^{13}C$, and DEPT-135 NMR data showed the presence of four singlet methyl groups (including a methoxy unit), six methylene groups, two methine groups, and nine quaternary carbons, including two oxygenated carbons in the aliphatic region and three carbonyl groups. These data accounted for all of the protons, indicating the absence of a free hydroxyl group. Importantly, the lack of any olefinic moieties required the presence of six rings to satisfy the degrees of unsaturation.

Analysis of the COSY data revealed two major spin systems. One of these comprised $H_2$-1/$H_2$-2/$H_2$-3, which was extended to form the western part of the structure through HMBC studies (Figure 1A). For example, $H_2$-1 displayed
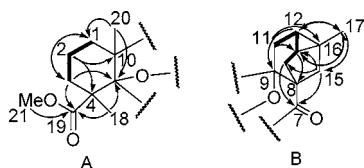


**Figure 1.** Partial structures of compound **1** illustrating key COSY (—) and HMBC (→) correlations.

HMBC correlations with C-2, C-3, and C-10, while the methyl protons of $H_3$-20 showed correlations with C-1, C-5, and C-10; the combination of these data construed the connections of both C-1 and C-20 to C-10 and then C-10 to C-5. A methoxycarbonyl group was inferred based on the chemical shifts of C-19 and C-21 along with a relevant HMBC correlation ($H_3$-21 to C-19). Also, the methyl protons of $H_3$-18 displayed HMBC correlations with C-3, C-4, C-5, and C-19, and, in conjunction with an HMBC correlation from $H_2$-3 to C-19, these data permitted the connectivities of both the C-18 methyl group and the C-19 methoxycarbonyl moiety to the quaternary C-4. The other spin system deduced from the COSY spectrum corresponded to the $H_2$-11/H-12/H-13/$H_2$-14 unit, and these were confirmed in turn via HMBC correlations (Figure 1B). Positions C-8, C-9, C-16, and C-17 were then incorporated with this four-carbon unit, based on HMBC correlations from $H_2$-11 to C-9 and

C-16, from H-12 to C-9 and cross correlations to position C-17, and from H-13 to C-8 and cross correlations to position C-17. At this point, an isolated methylene unit displaying an AB pattern ($H_2$-15; geminal coupling of 12 Hz) was positioned between the two quaternary carbons C-8 and C-16 based upon HMBC correlations from $H_3$-17 to C-15 and from $H_2$-15 to C-13 and C-14. Furthermore, both $H_2$-14 and $H_2$-15 showed HMBC correlations with C-7 ($\delta_C$ 198.6), one of the two remaining carbonyl carbons, thus requiring attachment of C-7 to C-8. The two partial structures in Figure 1 were united by connecting C-9 to C-10, and this was supported by key HMBC correlations of both $H_2$-1 and $H_3$-20 with C-9 and of $H_2$-11 with C-10.

The remaining assignments were for the other carbonyl unit (C-6; $\delta_C$ 190.0) and a single oxygen atom. The downfield chemical shift of both C-5 ($\delta_C$ 89.0) and C-9 ($\delta_C$ 87.3) suggested oxygenation, and thus, an oxetane ring could be proposed to share this single oxygen atom. The relatively upfield chemical shift of both of the C-6 and C-7 carbonyl moieties suggested an adjacent diketone configuration.[11,12] This assignment was supported by the lack of relevant HMBC correlations to C-6, given its position more than four bonds from any proton.

The relative stereochemistry of **1** was construed from analysis of molecular models, energy minimized using the MMFF94 force field in Spartan '04 (Wavefunction, Inc., Irvine, CA) overlaid with key correlations observed in the ROESY NMR spectrum (Figure 2). The oxetane ring and
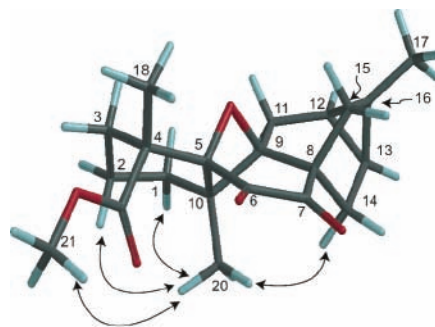


**Figure 2.** Energy minimized model of **1** illustrating the major ROESY correlations used to define the relative stereochemistry.

the adjacent diketone moieties impart considerable strain to this rigid ring system. Position 10 was pivotal to the assignment of the relative stereochemistry, as strong ROESY correlations were observed to $H_3$-20 from $H_3$-21, $H_2$-2, and $H_2$-14, which were all supported by calculated interatomic distances of approximately 2.4 Å. Position C-4 was determined by strong correlations from $H_3$-21 to $H_3$-20, also supported by a calculated interatomic distance of 2.4 Å from the molecular model, and although not conclusive, a ROESY

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**Table 1.** NMR Data for Mitrephorone A (1)[a]

| position | $\delta_C$ | DEPT | $\delta_H$ (mult.; $J$ in Hz) | HMBC (H → C) |
|---|---|---|---|---|
| 1 | 32.4 | $CH_2$ | 2.20 (m); 1.63 (m) | 2, 3, 9, 10 |
| 2 | 17.5 | $CH_2$ | 1.58 (m) | 1, 3, 4, 10 |
| 3 | 35.0 | $CH_2$ | 1.78 (br d; 10); 1.64 (br d; 10) | 1, 4, 5, 19 |
| 4 | 43.0 | C | | |
| 5 | 89.0 | C | | |
| 6 | 190.0 | C | | |
| 7 | 198.6 | C | | |
| 8 | 59.0 | C | | |
| 9 | 87.3 | C | | |
| 10 | 48.5 | C | | |
| 11 | 26.4 | $CH_2$ | 2.29 (dd; 17, 2); 2.11 (dd; 17, 2) | 8, 9, 10, 12, 13, 16 |
| 12 | 17.8 | CH | 0.74 (br dd; 7, 2) | 9, 17 |
| 13 | 20.9 | CH | 1.07 (dd; 7, 3) | 8, 17 |
| 14 | 30.1 | $CH_2$ | 2.49 (dd; 13, 3); 1.74 (d; 13) | 7, 8, 9, 12, 13 |
| 15 | 40.0 | $CH_2$ | 2.31 (d; 12); 1.63 (d; 12) | 7, 8, 9, 13, 14 |
| 16 | 21.6 | C | | |
| 17 | 19.1 | $CH_3$ | 1.21 (s) | 12, 13, 15, 16 |
| 18 | 18.4 | $CH_3$ | 1.12 (s) | 3, 4, 5, 19 |
| 19 | 174.9 | C | | |
| 20 | 18.3 | $CH_3$ | 1.09 (s) | 1, 5, 9, 10 |
| $OCH_3$-21 | 51.7 | $CH_3$ | 3.64 (s) | 19 |

[a] All NMR experiments in Tables 1 and 2 were performed in $CDCl_3$ with TMS as an internal standard; gs-COSY, ROESY, gs-HSQC, gs-HMBC, and ^{1}H NMR spectra were acquired using a Varian Unity Inova-500 instrument, while a Bruker DPX-300 instrument was utilized for some of the ^{13}C and DEPT-135 NMR spectra.

**Table 2.** NMR Data for Mitrephorones B (2) and C (3)

| | mitrephorone B (2) | | mitrephorone C (3) | |
|---|---|---|---|---|
| position | $\delta_C$ | $\delta_H$ (mult.; $J$ in Hz) | $\delta_C$ | $\delta_H$ (mult.; $J$ in Hz) |
| 1 | 30.5 | 1.19 (m); 1.69 (br d; 9) | 48.4 | 2.08 (d; 18); 2.53 (d; 18) |
| 2 | 14.5 | 1.74 (br d; 11) | 207.1 | |
| 3 | 30.7 | 1.54 (br d; 11); 2.18 (m) | 44.5 | 2.44 (d; 18); 2.96 (d; 18) |
| 4 | 45.5 | | 46.2 | |
| 5 | 141.3 | | 137.4 | |
| 6 | 142.3 | | 142.5 | |
| 7 | 197.9 | | 197.4 | |
| 8 | 48.3 | | 48.1 | |
| 9 | 48.0 | 1.78 (br d; 11) | 47.4 | 1.95 (br d; 11) |
| 10 | 37.3 | | 38.5 | |
| 11 | 19.9 | 1.86 (br dd; 9, 14); 2.02 (ddd; 4,11, 14) | 19.7 | 1.85 (br dd; 9, 12); 2.05 (br dd; 4, 12) |
| 12 | 19.1 | 0.71 (br d; 6) | 18.9 | 0.76 (br dd; 3, 7) |
| 13 | 22.4 | 1.02 (dd; 3, 6) | 22.3 | 1.07 (dd; 3, 8) |
| 14 | 33.7 | 1.91 (br d; 12); 2.18 (m) | 33.7 | 1.99 (m); 2.19 (br d; 12) |
| 15 | 47.4 | 1.47 (d; 12); 2.21 (d; 12) | 47.4 | 1.50 (d; 12); 2.25 (d; 12) |
| 16 | 24.1 | | 24.3 | |
| 17 | 19.9 | 1.22 (s) | 19.8 | 1.24 (s) |
| 18 | 22.6 | 1.45 (s) | 23.4 | 1.46 (s) |
| 19 | 177.2 | | 174.9 | |
| 20 | 21.5 | 1.33 (s) | 22.9 | 1.44 (s) |
| $OCH_3$−21 | 52.2 | 3.65 (s) | 52.8 | 3.70 (s) |
| OH-6 | | 6.24 (s) | | 6.32 (s) |

correlation was not observed between $H_3$-18 and $H_3$-20. The configurations for C-8 and C-13 were determined by ROESY correlations between $H_2$-14 and $H_3$-20. Moreover, the literature on *ent*-trachylobanes supports this configuration for the cyclopropane portion of these diterpenoids, including the configurations for C-12 and C-16.[13] Finally, the formation of the oxetane ring necessitates the displayed orientation of C-5 to C-9.

The trivial name mitrephorone A (1) was ascribed to this structure, which was consistent with the *ent*-trachylobane-type of plant metabolites,[13,14] a group of diterpenoids possessing a characteristic cyclopropane ring system. This new compound, however, expands the functionalities of this core structural type by including both an unprecedented C-5, C-9 oxetane ring system and the adjacent diketone moieties.

Comparison of the NMR data for compound 2 with those of 1 revealed structural similarities, including the presence of the upfield H-12 signal as exhibited by all other *ent*-trachylobanes.[10,13,14] The NMR and HRMS data for 2 revealed its molecular formula as $C_{21}H_{28}O_4$, which has one less degree of unsaturation than 1. In this case, an oxetane ring was not present, as evident from the absence of any oxygenated aliphatic carbons, and one of the two ketone

carbons of 1 (C-6) was replaced by an olefinic unit (C-5, $\delta_C$ 141.3; C-6, $\delta_C$ 142.3). Key HMBC correlations of $H_2$-14 and $H_2$-15 with C-7, and the C-6-OH proton with C-5, C-6, and C-7, permitted assignment of the structure of mitrephorone B as shown in 2.

The NMR data for compound 3 were comparable to those of 2, and this suggested a common core structure. In fact, the major differences between 2 and 3 were merely the presence of a new downfield ketone carbon (C-2; $\delta_C$ 207.1) and two pairs of isolated methylene units ($H_2$-1 and $H_2$-3) in 3. These data, together with the mass spectrometric information, suggested that the C-2 methylene in 2 was replaced by a carbonyl carbon in 3, and this was supported by HSQC and HMBC analyses. All protons of $H_2$-1 and $H_2$-3 showed strong HMBC correlations with C-2, making the unambiguous assignment of the structure of 3, as shown.

The relative stereochemistries of mitrephorone B (2) and mitrephorone C (3) were established independently based on similar ROESY data analysis as described for 1, and all three adopt analogous configurations as shown. The absolute stereochemistry for all three compounds was presumed to belong to their *enantio*- series, as determined by crystallographic analysis[9] and chemical transformations[10] of other known *ent*-trachylobane-type diterpenoids.

For the bioactivity-directed fractionation studies, the human KB carcinoma assay was used,[15,16] and all pure compounds were evaluated also using a human cancer cell

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**Table 3.** Cytotoxicity and Antimicrobial Activity of Compounds Isolated from *Mitrephora glabra*

| | IC$_{50}$ Values ($\mu$g/mL)$^a$ | | | | Minimal Inhibitory Concentration ($\mu$g/mL)$^b$ | | | |
|---|---|---|---|---|---|---|---|---|
| compound | KB | MCF-7 | H460 | SF-268 | *M. luteus* | *M. smegmatis* | *S. cerevisiae* | *A. niger* |
| **1** | 8.0 | 15.7 | 23.3 | 30.9 | 125 | 63 | 63 | 63 |
| **2** | 7.0 | inactive | inactive | inactive | 88 | 88 | 88 | 88 |
| **3** | inactive$^c$ | inactive | inactive | inactive | 63 | 31 | 31 | 63 |
| **4** | inactive | inactive | inactive | inactive | 19 | 75 | 75 | 75 |
| positive controls$^d$ | 0.005 (camptothecin) | 0.025 (camptothecin) | 0.002 (camptothecin) | 0.021 (camptothecin) | 0.1 (ampicillin) | 0.2 (kanamycin) | 25 (amphotericin B) | 25 (amphotericin B) |

$^a$ KB: human oral epidermoid carcinoma; MCF-7: human mammary adenocarcinoma; H460: human nonsmall cell (large cell) lung carcinoma; SF-268: human astrocytoma. IC$_{50}$ values are determined as the concentration required to reduce cellular staining with sulforhodamine B by 50% relative to untreated controls following 72 h of continuous exposure.[17] $^b$ Minimal inhibitory concentration is the lowest concentration of compound completely inhibiting growth (as turbidity) expressed in $\mu$g/mL.[17] $^c$ Inactive = IC$_{50}$ value > 30 $\mu$g/mL. $^d$ Average values.

panel of MCF-7, NCI-H460, and SF-268 cell lines.[17] Of the three new compounds, **1** displayed the broadest spectrum of cytotoxicity, having a modest IC$_{50}$ value against all cell lines (Table 3), even SF-268, a slow-growing tumor cell line. Compound **2** had modest activity against KB cells, but was inactive otherwise, while compound **3** lacked discernible cytotoxicity against any of the four cancer cell lines.

Trachylobane diterpenoids are reported to have antimicrobial activity.[3,13,18] As such, the minimal inhibitory concentration (MIC) of all the compounds was measured against bacteria (*Micrococcus luteus* and *Mycobacterium smegmatis*), a yeast (*Saccharomyces cerevisiae*), and a filamentous fungus (*Aspergillus niger*).[17] Compounds **1–3** exhibited modest MIC values and were equipotent against all organisms. This is in sharp contrast to the anticancer data, where only compound **1** had broad-spectrum activity (Table 3). Moreover, the least active compound in the anticancer assays, compound **3**, appeared to have the strongest antimicrobial activity of the three, possessing an MIC value against the yeast, *S. cerevisiae*, that was similar to that of the standard antifungal antibiotic, amphotericin B.

In summary, this is the first reported investigation of *Mitrephora glabra* for bioactive compounds, and three new *ent*-trachylobane-type diterpenoids, mitrephorones A–C (**1–3**), were obtained. The oxetane ring in mitrephorone A (**1**) was not present in any of the known members of this class, making it the first representative of this novel hexacyclic ring system. On the basis of a limited set of human tumor cell cytotoxicity data, this unique structure seems to impart a greater degree of anticancer activity than in the other trachylobanes. In the antimicrobial assays, all of the compounds had similar levels of activity, regardless of the presence or absence of the oxetane ring.

**Supporting Information Available:** Experimental details and 1- and 2-D NMR spectra of mitrephorone A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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