Use of On-flow LC/^{1}H NMR for the Study of an Antioxidant Fraction from *Orophea enneandra* and Isolation of a Polyacetylene, Lignans, and a Tocopherol Derivative

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Received May 15, 1998

The CH_2Cl_2 extract of the leaves of *Orophea enneandra* displayed antifungal, antioxidant, and radical scavenging properties in bioautographic TLC assays. To obtain rapid information on the active compounds, on-flow LC/^{1}H NMR and LC/UV/MS analyses of the antioxidant fraction were performed. The on-line information led rapidly to the partial identification of three closely related lignans, one tocopherol derivative, and one polyacetylene. This approach necessitated, however, large quantities to be injected to obtain satisfactory on-flow LC/^{1}H NMR spectra, and isolation of the compounds was necessary to obtain complete NMR data. These compounds were isolated and identified as (−)-phylligenin (**1**), (−)-eudesmin (**2**), (−)-epieudesmin (**3**), polycerasoidol (**4**), and oropheic acid (**5**), a new polyacetylene. Their activities against the 2,2-diphenyl-1-picrylhydrazyl radical and the fungus *Cladosporium cucumerinum* were investigated. This paper indicates the possibilities and limits of on-flow LC/^{1}H NMR in this type of study.

In the course of our systematic studies on Indonesian plants used in traditional medicine, we have investigated *Orophea enneandra* Bl. This small tree belongs to the Annonaceae and is distributed throughout Java.[1] Although no medical uses for *O. enneandra* are directly reported in the traditional pharmacopoeia of Indonesia, it is known that other species of the same genus are used to treat different ailments (fever, cough, etc.).[2] To our knowledge, there has been no report on the phytochemical investigation of *O. enneandra*. Thus, the leaves of this plant were dried and successively extracted with dichloromethane and methanol at room temperature. The dichloromethane extract displayed in bioautographic TLC assays antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum*[3] and antioxidant and radical scavenging properties toward β-carotene[4] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical,[5] respectively. To obtain rapid information on the active compounds, on-flow LC/^{1}H NMR and LC/UV/MS analyses of the antioxidant fraction were performed. The on-line information quickly led to the partial identification of three closely related lignans, one tocopherol derivative, and one polyacetylene. We report here on the on-line spectroscopic data obtained from the on-flow LC/^{1}H NMR and LC/UV/MS, on the possibilities and limits of on-line identification by LC/NMR analysis and on the isolation; structure elucidation; and antioxidant, free radical scavenging, and antifungal activities of the five main constituents.

Results and Discussion

Dried and powdered leaves of *O. enneandra* were extracted successively with dichloromethane and methanol. The dichloromethane extract (5.0 g) was fractionated on a MPLC Si gel column with a step-gradient of petroleum ether−ethyl acetate to afford eight fractions (I−VIII). Fraction IV exhibited antioxidant and free radical scavenging activities. The LC/UV analysis of this fraction enabled the detection of five main constituents (**1**−**5**). For the determination of the compounds responsible for the antioxidant activity, fractions were directly collected every two minutes from an analytical HPLC separation and were submitted to autographic assays (see Experimental Section). LC/UV peaks **1** and **4** were thus assigned with precision as the antioxidant compounds.

To obtain rapid preliminary structure information on these active constituents, a chemical screening by LC/UV/MS and LC/NMR was performed on the antioxidant fraction. The UV spectrum of **1** was typical of lignans (nm: 230, 280),[6] while that of **4** could not be directly linked to a class of compound (nm: 234, 294). The LC/TSP-MS analysis indicated for **1** and **4** molecular weights of 372 and 358 amu revealed by peaks at *m*/*z* 373, 390 and 359, 376 ($[M + H]^+$, $[M + NH_4]^+$), respectively.

For the LC/NMR analysis, the same LC conditions as for LC/UV/MS were used, except that the water was replaced by D_2O. An LC/NMR solvent suppression sequence (WET), consisting in a combination of laminar shifted shaped selective pulses associated with dephasing gradients and selective ^{13}C decoupling,[7] was run before each acquisition in order to efficiently remove the residual HOD signal and the resonances of acetonitrile and its two ^{13}C satellites. The amount of the antioxidant fraction injected for this LC/NMR had to be consequently increased (2 mg on column) in order to obtain good quality on-flow LC/^{1}H NMR spectra of the five main constituents **1**−**5** (Figures 1 and 2).

The on-flow LC/^{1}H NMR of **1** showed different signals that could be assigned to a lignan skeleton. The aliphatic region was, however, difficult to resolve. The analysis of two other closely related lignans (**2** and **3**) was necessary to determine a possible structure for **1**. The UV spectra of **1**−**3** were similar, and the TSP-MS for both **2** and **3** indicated a molecular weight of 386 amu revealed by peaks at *m*/*z* 387 ($[M + H]^+$) and 404 ($[M + NH_4]^+$). The on-flow LC/^{1}H NMR of **2** showed only a few signals, implying the presence of a highly symmetrical compound. Two singlets at δ 3.86 (6H) and δ 3.88 (6H) due to aromatic methoxy

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10.1021/np980203p CCC: $15.00 © 1998 American Chemical Society and American Society of Pharmacognosy
Published on Web 10/29/1998

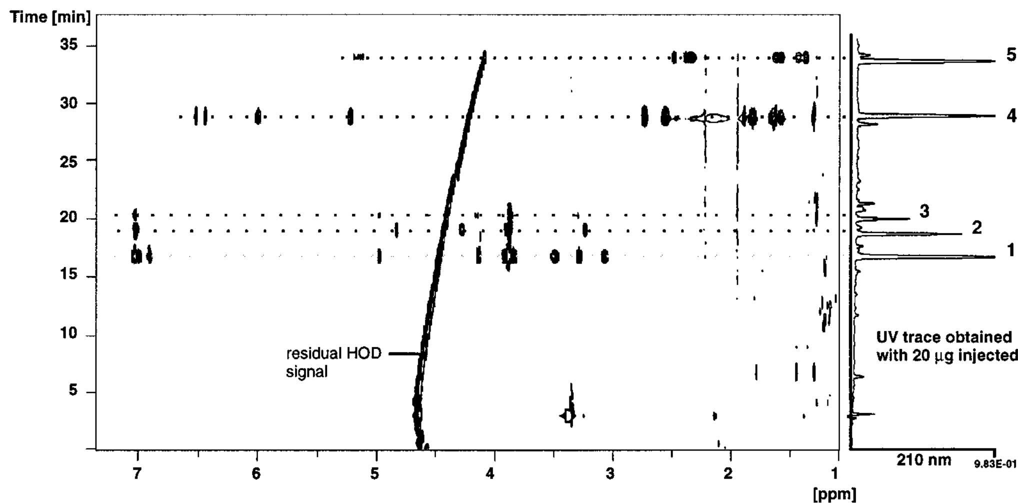


Figure 1. On-flow 2D LC/^{1}H NMR chromatogram of the antioxidant fraction from the CH_2Cl_2 extract of *Orophea enneandra* on NovaPak RP-18 (150 × 3.9 mm i.d., 4 μm), MeCN−D_2O gradient (20:80 to 95:5 in 50 min, 1 mL/min), 0.05% TFA, 2 mg injected. UV trace was recorded in the same conditions with 20 μg injected. The residual signal of HOD is indicated.

groups, six aromatic protons in a narrow region between δ 7.02 and 7.03, and eight aliphatic protons at δ 3.23 (br s, 2H), 3.90 (s, 2H), 4.27 (t, J = 7 Hz, 2H), and 4.83 (br s, 2H) suggested the presence of a lignan with two aromatic rings having each two methoxy groups. The TSP-MS, and on-flow ^{1}H NMR spectra suggested **2** to be eudesmin by comparison with literature data.[6]

The on-flow LC/^{1}H NMR of compound **3** was comparable to that of its isomer, **2**, except for the aliphatic protons that exhibited signals at δ 3.05 (m, 1H), 3.29 (t, J = 8 Hz, 1H), 3.49 (m, 1H), 3.84 (m, 1H), 3.91 (t, J = 7 Hz, 1H), 4.15 (d, J = 10 Hz, 1H), 4.55 (d, J = 8 Hz, 1H), and 4.97 (d, J = 7 Hz, 1H). This showed that the configuration at C_6 was opposite that of **2**. Thus, **3** was tentatively identified as epieudesmin by reference to literature data for lignans.[6,8]

The on-flow LC/^{1}H NMR of the antioxidant compound **1** (Figure 2) was very similar to that of **3**, except that only three methoxy groups were observed. This confirmed that the chemical shifts of the on-flow ^{1}H NMR spectra of **1** and **3**, together with a difference of 14 amu between the LC/MS of these compounds, suggested **1** to be phylligenin, a lignan similar to epieudesmin with one methoxy group less.[6]

The on-flow LC/^{1}H NMR of the second antioxidant compound **4** (Figure 2) exhibited three signals at δ 1.30 (s, 3H), 1.62 (s, 3H), and 1.89 (s, 3H) due to methyl groups, two aromatic protons at δ 6.44 (s, 1H) and 6.52 (s, 1H), two olefinic protons at δ 5.22 (t, J = 7 Hz, 1H) and 6.00 (t, J = 7 Hz, 1H), and eight aliphatic protons at δ 1.60 (m, 2H), 1.81 (m, 2H), 2.56 (dd, J = 8,7 Hz, 2H), and 2.74 (t, J = 7 Hz, 2H). With these on-line data, it was difficult to make any suggestion for the possible structure of **4**. However, a cross-check of its molecular weight (358 amu) with all known constituents of the Annonaceae family in a database of natural products[9] suggested **4** to be polycerasoidol, a tocopherol derivative isolated from *Polyalthia cerasoides* (Annonaceae).[10] The ^{1}H NMR of poylcerasoidol was comparable to the on-flow ^{1}H NMR of **4**. However, the chemical shifts of the on-flow ^{1}H NMR of **4** between 1.90 and 2.10 ppm were not visible due to their location under the supressed peaks of the LC-solvent system (CH_3-CN, δ 1.90−2.10).[11]

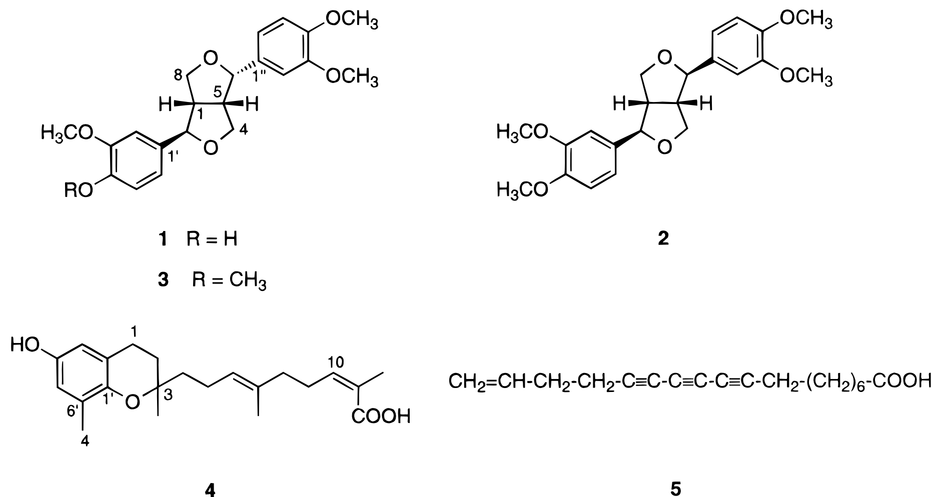
To quantify the antioxidant properties of **1** and **4** in a dilution assay, their targetted isolation was undertaken. During this procedure, compounds **2** and **3** were also obtained. Compounds **1**−**4** were finally identified as (−)-phylligenin, (−)-eudesmin, (−)-epieudesmin, and polycerasoidol, respectively, from their spectroscopic data (UV, EIMS, D/CIMS, ^{1}H and ^{13}C NMR),[6,8,10] confirming the structural assignments made according to the on-flow data.

Antioxidative and radical scavenging properties of compounds **1** and **4** were evaluated toward β-carotene[4] and DPPH radical,[5] respectively. Antioxidative properties were determined by bleaching experiments. On TLC plates, 10 μg of **1** and **4** inhibited the bleaching of β-carotene, while the same amount of each compound reduced the DPPH radical. Compounds **1** and **4** were also tested against DPPH in a spectrophotometric assay. Quercetin, BHT [2,6-di(*tert*-butyl)-4-methylphenol], and cinnamic acid were used as reference compounds. Activities of **1** and **4** were weaker than that of quercetin but higher than that of BHT. Among the isolated compounds, polycerasoidol exhibited the strongest activity. The scavenging property of **4** on the DPPH radical was similar to that of α-tocopherol (Figure 3).

The LC/UV of the antioxidant fraction IV from the dichloromethane extract displayed for compound **5** a typical UV spectrum of polyacetylenes,[13] exhibiting maxima at 204 and 212 nm and low intensity bands at 253, 267, 282, and 310 nm. While acetylenic compounds have been isolated from *Porcelia macrocarpa*,[14] there has been no report of polyacetylenes in the Annonaceae. Thus, we were interested to identify this compound. Its TSP-MS spectrum presented ion at m/z 288, indicating a molecular weight of 270 amu. The on-flow ^{1}H NMR of **5** (Figure 2) exhibited two olefinic signals at δ 5.15 (m, 2H), and 5.91 (m, 1H) and 18 aliphatic protons at δ 2.49 (t, J = 7 Hz, 2H), 2.39 (t, J = 7 Hz, 2H), 2.34 (dd, J = 8, 15 Hz, 2H), 1.60 (t, J = 7 Hz, 2H), 1.57 (m, 2H), 1.43 (m, 2H), 1.38 (m, 2H), and 1.36 (m, 2H). As the comparison of the on-flow data of **5** with the literature data of polyacetylenes did not allow a positive identification, a targetted isolation of **5** was undertaken.

Compound **5** appeared to be rather unstable, and there was some degradation into a blue compound insoluble in methanol. This degraded product was discarded, and spectroscopic measurements of pure **5** were consistent with the data obtained on-line.

The molecular formula, $C_{18}H_{22}O_2$, of compound **5** was deduced from the ^{13}C NMR and DEPT spectra and confirmed by D/CIMS (ammonium adduct ion $[M + NH_4]^+$ at m/z 288). As mentioned above, the UV spectrum showed characteristic absorption bands, indicating the presence of



a polyacetylene. The very low ε values of the low intensity bands indicated that the triple bonds were not conjugated with double bonds. The ^{13}C NMR and DEPT spectra of **5** showed signals for six quaternary carbons (δ 80.42, 79.42, 66.59, 66.17, 61.14, 60.80), characteristic of three consecutive triple bonds with two neighboring methylene groups (δ 19.78 and 19.75),[13] a methylene (δ 38.10) adjacent to a carboxyl group (δ 181.41), and a methylene (δ 33.32) next to a vinyl group (δ 137.47 and 116.56). The ^{1}H NMR spectrum of **5** recorded in MeOD ressembled that obtained by on-flow LC/^{1}H NMR in MeCN−D_2O. Most of the signals were just slightly shifted, due to the solvent change. However, one new resonance at 2.19 ppm, not visible in the on-flow LC/^{1}H NMR due to its location under the supressed peaks of the LC-solvent system, was recorded. The ^{1}H NMR spectrum of **5** in MeOD exhibited two signals at δ 5.09 (dd, J = 1.5, 17 Hz, 1H) and 5.04 (dd, J = 1.5, 10 Hz, 1H), corresponding to a terminal vinylic methylene group, and a signal at δ 5.83 (m, 1H), due to its remaining olefinic proton. The methylene group next to the carboxylic acid gave a triplet at δ 2.19 (J = 7.6 Hz, 2H) due to its coupling with one aliphatic CH_2 group. This was confirmed by a ^{1}H−^{1}H COSY experiment. Triplets at δ 2.39 (J = 7.0 Hz, 2H) and 2.30 (J = 7.0 Hz, 2H) were observed in the ^{1}H NMR experiment spectrum due to the methylene groups on both sides of the triple bond system; these were also coupled to aliphatic CH_2 groups. 2D ^{1}H−^{1}H COSY and ^{1}H−^{13}C HSQC−TOCSY NMR experiments revealed the partial structures CH_2=CH−CH_2−CH_2−C≡ and ≡C−CH_2−$(CH_2)_6$−COOH. The correlations of the proton signals to their respective carbons were achieved by a ^{1}H−^{13}C HSQC experiment. The long-range ^{1}H−^{13}C HMBC spectrum indicated clearly the correlations between the CH_2 and the methynes, proving the presence of a −CH_2−C≡C−C≡C−C≡C−CH_2− system (Figure 4). It confirmed also the terminal position of the carboxyl group at δ 181.4. Thus, the structure of polyacetylene **5** was established as octadeca-17-en-9,11,13-triynoic acid, and this compound was named oropheic acid.

In our general screening procedures, compounds **1**−**4** showed antifungal activity against the phytopathogenic fungus *Cladosporium cucumerinum*.[3] The minimum quantities spotted on TLC plates required to inhibit the growth of *C. cucumerinum* were 20 μg for compounds **1**−**3** and 40 μg for **4**. Miconazole, used as positive control, was active at 10 μg.

In conclusion, the present work showed that LC/NMR associated with LC/MS was a very helpful technique for the preliminary structure assignment of the main constituents of the antioxidant fraction. However, direct LC/NMR measurements of crude extracts are not always possible if the compounds of interest are present in small amounts, due to the relatively low sensitivity of the method. This problem could be partially avoided by performing stop-flow analyses.[15] When working with unknown compounds, the potential presence of signals hidden under the solvent-suppressed peak(s) should be taken in consideration. If all signals have to be recorded, reinjection of the sample using a different elution system should be performed. Another point to take into consideration is that the on-flow LC/^{1}H NMR data were substantially different from those of the isolated compounds because the NMR solvent for on-flow LC/^{1}H NMR is a mixture of MeCN−D_2O, while that of the isolated compounds was one solvent. The example described here illustrates well this on-flow LC/NMR approach, but the relatively low sensitivity of the method and the problems due to the suppression of the solvent mean that LC/NMR is probably not yet ready to become a routine part of plant-screening analysis. However, this hyphenated method has been very recently introduced in the field of natural products, and important technical developments are expected in the near future, which should improve substantially its sensitivity and ease of use.[16]

Lignans are not common in the Annonaceae, they have been found only in the genus *Rollinia*.[17,18] This is the first report on the genus *Orophea*. The tocopherol derivative, polycerasoidol, was reported to occur in one other plant, *Polyalthia cerasoides*, also in the Annonaceae family.[10] Furthermore, previous to this work, no polyacetylenes have, to our knowledge, been described in the Annonaceae.

Experimental Section

General Experimental Procedures. TLC: Si gel 60 F_{254} Al sheets (Merck), detection with Godin reagent.[19] The solvent system employed for all the TLC assays was petroleum ether−EtOAc (1:2). MPLC: home-packed Si gel column (63−200 μm, Merck; 450 × 26 mm i.d.) and Lichroprep RP-18 column (15−25 μm; 460 × 16 mm i.d.). UV: Perkin−Elmer Lambda-20 spectrophotometer. Spectra were recorded in MeOH. Optical rotation: Perkin−Elmer 241 MC polarimeter. Microplate reader: Spectro-Rainbow instrument. Analytical HPLC: Hewlett−Packard 1050 instrument equipped with a photodiode array detector. ^{1}H and ^{13}C NMR spectra were measured on a Varian Innova Unity 500 spectrometer at 500 and 125 MHz, respectively. TMS was used as internal standard.

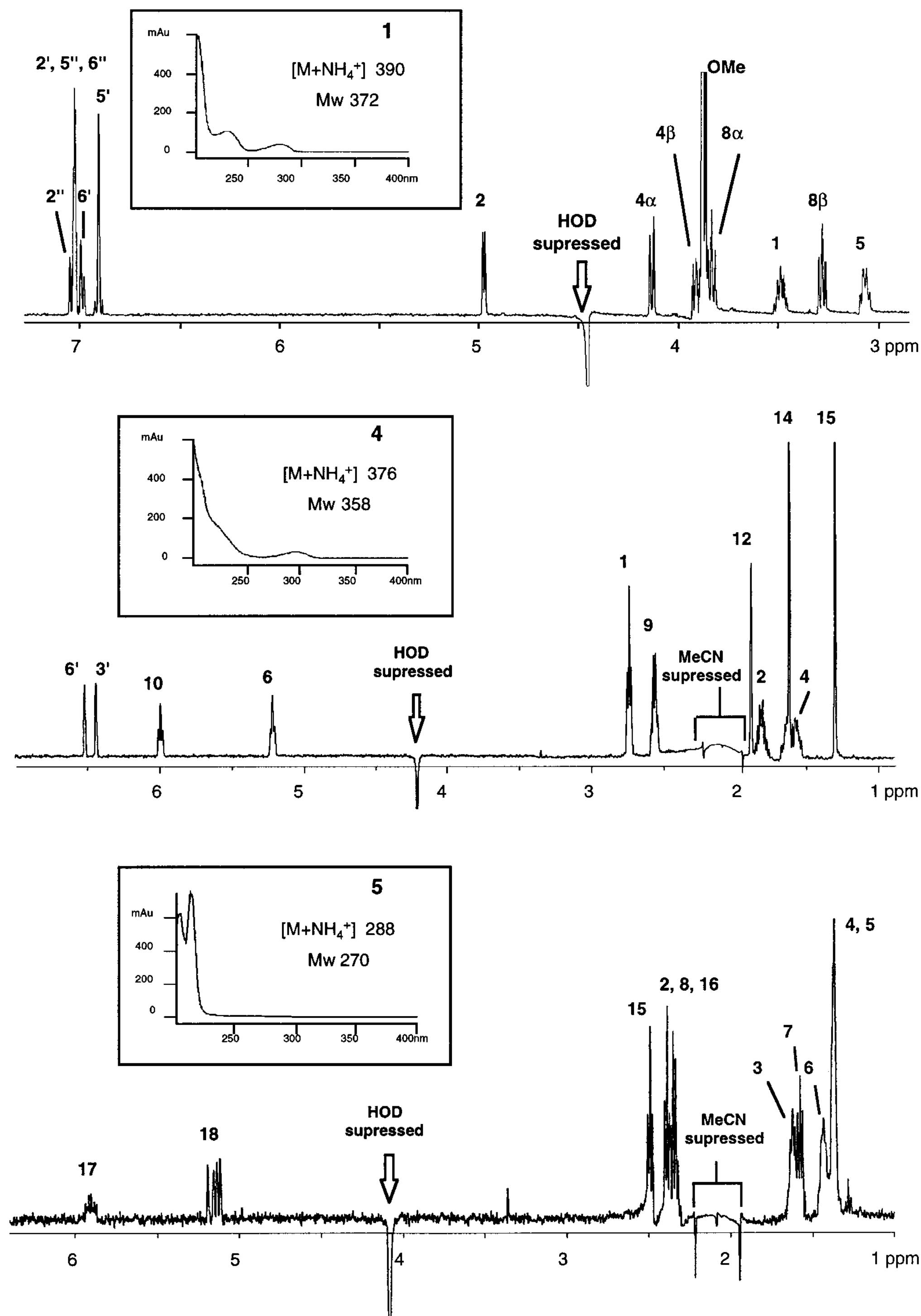


Figure 2. LC/UV/MS and on-flow LC/^{1}H NMR spectroscopic data of the two antioxidant compounds (**1**, **4**) and the new polyacetylene (**5**).

EIMS and D/CIMS: Finnigan MAT TSQ-700 triple stage quadrupole instrument. LC/UV/MS: Waters 600-MS solvent delivery system; Waters-NovaPak RP-18 column (4 μm; 150 × 3.9 mm i.d.); Hewlett–Packard 1050 photodiode array detector; Finnigan MAT TSQ-700 triple stage quadrupole instrument with a Thermospray 2 interface; 20 μg of the fraction IV were injected. LC/UV/NMR: Varian-Innova-Unity 500 spectrometer equipped with a ^{1}H[^{13}C] pulse-field gradient indirect detection microflow LC/NMR probe (flow cell 60 μL; 3 mm i.d.). Reversed-phase HPLC of the enriched fraction was carried out on a Varian modular HPLC system, composed of a Varian 9012 pump, a Valco injection valve, and a Varian 9050 UV detector. The separation was performed by using a Waters–Nova-Pak RP-18 column (4 μm; 150 × 3.9 mm i.d.) with linear gradient of MeCN–D_2O (from 20:80 to 95:5 in 50 min); 2 mg of the fraction IV were injected. Reference of the LC solvent signals was set at δ 2.10 for MeCN.

Plant Material. Leaves of *O. enneandra* were collected on Sepu Island, East Java, Indonesia, in August 1993, and were identified by Miss Afriastini. Voucher specimens have been deposited at the National Herbarium of Bogor, Indonesia, and at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland (no. 93051).

Fractionation by Analytical HPLC. The antioxidant fraction (200 μg) was injected on a HPLC with a linear gradient of MeCN–H_2O from 20:80 to 95:5 in 50 min. Fractions were collected every two minutes. These were evaporated and diluted with 100 μL of MeOH. Fractions, 10 μL each, were

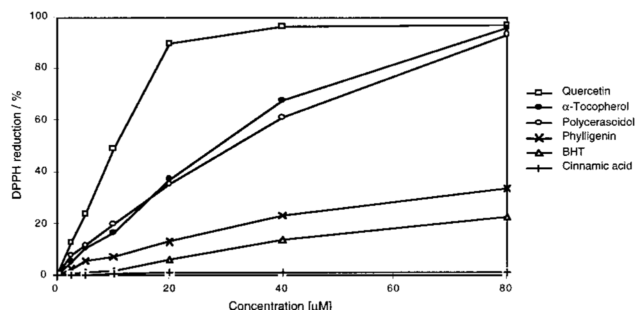


Figure 3. Scavenging activities of phylligenin (**1**) and polycerasoidol (**4**) on DPPH radical compared to those of quercetin, BHT, cinnamic acid, and α-tocopherol. Measurement at 517 nm, determination after 30 min.

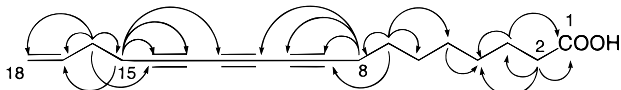


Figure 4. Selected long-range $^{1}H-^{13}C$ couplings observed for **5** in a HMBC experiment.

spotted on a TLC plate and submitted to the β-carotene and the DPPH TLC assays.

Extraction and Isolation. Dried leaves (250 g) were ground and extracted at room temperature successively with CH_2Cl_2 and MeOH (3 × 2.5 L each). The CH_2Cl_2 extract (5.0 g) was fractionated by MPLC on a Si gel Si 60 column into 8 fractions (I −VIII) with a step-gradient elution of petroleum−EtOAc (2:1 to 2:10) and finally MeOH. Fraction IV (670 mg) was subjected to MPLC on RP-18 with a H_2O−MeCN gradient (7:3 to 7:9) and yielded **1** (60 mg), **2** (21 mg), **3** (12 mg), **4** (92 mg), and **5** (40 mg).

Octadeca-17-en-9,11,13-triynoic acid (5): unstable white amorphous powder; TLC R_f 0.40; UV (MeOH) (log ϵ) 204 (4.3), 212 (3.6), 253 (2.5), 267 (2.6), 282 (2.6), 310 (2.2) nm; ^{1}H NMR (CD_3OD, 500 MHz) δ 5.83 (1H, m, H-17), 5.09 (1H, dd, J = 1.5, 17 Hz, H-18 trans), 5.04 (1H, dd, J = 1.5, 10 Hz, H-18 cis), 2.39 (2H, t, J = 7.0 Hz, H-15), 2.30 (2H, t, J = 7.0 Hz, H-8), 2.27 (2H, dd, J = 7.0, 13.9 Hz, H-16), 2.19 (2H, t, J = 7.6 Hz, H-2), 1.60 (2H, m, H-3), 1.53 (2H, m, H-7), 1.40 (2H, m, H-6), 1.35 (2H, m, H-5), 1.34 (2H, m, H-4). ^{13}C NMR (CD_3-OD, 125 MHz) δ 181.41 (C-1), 137.47 (C-17), 116.56 (C-18), 80.42 (C-9), 79.42 (C-14), 66.59 (C-13), 66.17 (C-10), 61.14 (C-11), 60.80 (C-12), 38.10 (C-2), 33.32 (C-16), 30.47 (C-4), 29.94 (C-5), 29.83 (C-6), 29.21 (C-7), 27.25 (C-3), 19.78 (C-15), 19.75 (C-15); D/CIMS (NH_3, positive ion mode) m/z 288 [M + NH_4^+] 100), 271 [M + H^+] (21), 254 (12), 219 (3), 180 (4); LC/TSP-MS data (NH_4OAc buffer, positive ion mode) m/z 288 [M + NH_4^+] (100), 271 $[M + H]^+$ (3), 231 (12), 182 (2).

Antifungal Assay. Bioautography with spores of *C. cucumerinum* for the estimation of antifungal activity was performed by the method of Homans and Fuchs.[3]

Antioxidant and Free Radical Scavenging Assays. The bleaching experiment on β-carotene was carried out on TLC plates. After developing and drying, the plates were sprayed with a 0.05% solution of β-carotene in $CHCl_3$. Plates were placed under UV 254 nm light until the background became discolored.

Reduction of DPPH Radical. TLC autographic assay: after developing and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. The plates were examined 30 min after spraying. Active compounds appear as yellow spots against a purple background. Spectrophotometric assay: the test was carried out on 96-well microplates; 50 µL of a 0.022% DPPH solution in MeOH were added to a solution of the compound to be tested in MeOH (230 µL). Absorbance at 517 nm was determined after 30 min, and the percentage of activity was calculated.

Acknowledgment. Financial support has been provided by the Swiss National Science Foundation.

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