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Anti-TB Polyynes from the roots of *Angelica sinensis*

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Abstract

Following chemotaxonomic evidence, the PE and CHCl$_3$ extracts of the roots of the botanical *Angelica sinensis* (Oliv.) Diels (Dang Gui) were investigated for *in vitro* anti-TB activity, in parallel to studying their serotonergic and GABAergic potential. The activities were confirmed to chemically overlap with the neurotropic active principles present in medium lipophilic fractions. Phytochemical investigations led to the isolation of five polyynes: falcarindiol (**1**), 9Z,17-octadecadiene-12,14-diyne-1,11,16-triol,1-acetate (**2**), oplopandiol (**3**), heptadeca-1-ene-9,10-epoxy-4,6-diyne-3,8-diol (**4**), and the new polyyne 8-hydroxy-1-methoxy-(*Z*)-9-heptadecene-4,6-diyn-3-one (**5**), as characterized by spectroscopic techniques including 1D, 2D NMR and HR-MS. All compounds were tested against two pathogenic strains of *Mycobacterium tuberculosis* (H37R*v* and ᴇʀᴅᴍᴀɴ) *in vitro* in a microplate Alamar Blue assay (MABA). The most potent anti-TB constituents were **1** and **2**, exhibiting MIC values of 1.4-26.7 μg/ml; **3** showed moderate MICs (49.5 and 50.2 μg/ml, respectively) while **4** and **5** were weakly active (MIC> 60 μg/ml). Notably, none of the five compounds exhibited significant cytotoxicity against VERO cells. These findings not only reveal a new potential area of therapeutic value for *A. sinensis*, but also underline the role of polyynes as anti-TB active principles in ethnobotanical preparations, and as lead compounds.

Keywords

Angelica sinensis; botanicals; dong quai; dang gui; polyynes; anti-TB activity; tuberculosis

INTRODUCTION

Every year approximately 2 million people die from curable tuberculosis (TB). Herbal medicines and other alternative therapies are increasingly used for TB treatment, especially for drug-resistant TB and multidrug-resistant tuberculosis (MDR-TB). *Angelica sinensis* (Oliv.) Diels (commonly called Dang Gui or Dong Quai) is a perennial apiaceous herb indigenous to northwest China. Its roots have been used for many diseases in Asia, especially for women's disorders, including anemia, dysmenorrhea, amenorrhea, premenstrual and

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menopausal syndromes (China Pharmacopoeia 2000). However, no previous study of *A. sinensis* for anti-TB activities has been reported.

Parallel to ongoing studies of the serotonergic and GABAergic potential of Dang Gui (Deng *et al.*, 2006a and 2006b), the present work followed chemotaxonomic evidence linked to potential anti-TB activity. Polyynes are characteristic triple-unsaturated natural products that have been found in seven plant families, i.e. Araliaceae, Campanulaceae, Asteraceae, Pittisporaceae, Oleaceae, Santalaceae and Apiaceae (Hansen *et. al.*, 1986), and are known to occur in *A.* species. These highly unsaturated natural products have previously been reported to exhibit general antifungal and antibacterial, but also anti-mycobacterial activities (Furumi *et. al.*, 1998; Kemp, 1978; Kobaisy *et. al.*, 1997). Preliminary investigation of the petroleum ether (PE) and chloroform ($CHCl_3$) extracts of Dang Gui roots confirmed the presence of anti-TB effects *in vitro*. These activities phytochemically overlapped with the neurotropic activities and were also concentrated in medium lipophilic fractions. The detailed overall phytochemical investigation of the PE and $CHCl_3$ Dang Gui extracts led to the isolation and identification of five polyynes (**1-5**). All polyynes **1-5** were tested for inhibitory activity toward the growth of *M. tuberculosis* H37R*v* and Erdman *in vitro* by using the MABA assay. In addition, **1-5** were evaluated for cytotoxicity against VERO cells and selectivity indices (SI) were determined as a measure for the drug lead potential of the compounds.

MATERIALS AND METHODS

General Methods and Plant collection

General materials and instrumentation were as previously described (Deng *et al.*, 2006b). The roots of *A. sinensis* were purchased in 2000 from Kiu Shun Trading Ltd., Vancouver, Canada and identified by the authors at UIC (Deng *et al.*, 2003). A voucher specimen is deposited at the UIC/NIH Center for Botanical Dietary Supplements Research, Chicago, IL, USA.

Extraction and Isolation

The detailed isolation procedures were performed as previously described. (Deng *et al.*, 2006b). Briefly, 8 kg of the pulverized roots of *Angelica sinensis* (Oliv.) Diels were extracted with methanol and the extract sequentially partitioned with PE, $CHCl_3$, and BuOH. The PE and $CHCl_3$, which contained the anti-TB activity, were combined and then fractionated by vacuum liquid chromatography (VLC). An aliquot (6.6 g) of subfraction A was further fractionated by repeated VLC now eluting with a gradient of PE-EtOAc-MeOH, and finally purified by reverse-phase (RP) preparative HPLC (75% MeOH in H_2O, 6 mL/min). This resulted in the isolation of the five polyynes **1** (48.1 mg, t_R = 73 min), **3** (1.6 mg, t_R = 91 min), **4** (1.4 mg, t_R = 46 min), and **5** (0.7 mg, t_R = 94 min). Another subfraction B (4.5 g) was re-chromatographed by VLC over a silica gel column with a stepwise gradient of hexane-$CHCl_3$-EtOAc (20:80:0 → 0:0:100), and then further purified by RP-C_{18} medium pressure liquid chromatography (MPLC) eluting with a MeOH-H_2O gradient from 50% MeOH in H_2O to 100% MeOH.). Finally, preparative thin-layer chromatography (TLC) (silica gel, 1 mm, 20 cm × 20 cm), developed with CH_2Cl_2-EtOAc (7:3), yielded compound **2** (8.7 mg, *Rf* = 0.6).

Anti-TB biological assays

Bacterial strains—For the preparation of the inocula of the two virulent strains of *M. tuberculosis*, H37R*v* (ATCC 27294) and Erdman (ATCC 35801) bacteria were grown in 100 ml of Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.2% (vol/vol) glycerol (Sigma Chemical Co., Saint Louis, Mo.), 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase; Difco), and 0.05% (vol/vol) Tween 80 (Sigma), also referred to as 7H9GC-T80.

Anti-TB Microplate Alamar blue Assay (MABA)—Anti-TB susceptibility testing of extracts and isolates was determined in the fluorometric Microplate Alamar Blue Assay (MABA) assay as described previously (Collins *et. al.*, 1997) in black, clear-bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence. Initial drug dilutions were prepared in DMSO, and subsequent twofold dilutions were performed in 0.1 ml of 7H12 medium in the microplates. Rifampin (RMP, Sigma) was used as a positive control, the solvent (DMSO) as a negative control.

For inoculation, both *M. tuberculosis* strains were diluted in 7H12 media to reach approximately 2×10^5 CFU mL^{-1}, and 0.1 ml was added to wells containing the test compounds in 100 μl of 7H12. Plates were incubated at 37 °C for 6 days. At day 7 of the incubation, 20 μl of Alamar Blue solution (Trek Diagnostic Systems, Cleveland, Ohio) and 12.5 μl of 20% Tween 80 were added to all of the wells, and the plates were re-incubated at 37 °C for 24 hours. Fluorescence was measured in a Victor II multilabel fluorometer (Perkin Elmer Life Sciences Inc., Boston, MA) at 530 nm and 590 nm. Thus, minimum inhibitory concentrations (MICs) were determined after incubation for 7 days at 37°C, defining percent inhibition as 1-(test well fluorescence units/mean FU fluorescence units of triplicate wells containing only bacteria) × 100. The MIC values refer to the concentration at which samples exhibited an inhibition of 90%.

Cytotoxicity assay—Evaluation of the cytotoxic activity of isolates in Vero cells (African green monkey kidney cells) was performed as described previously (Cantrell *et. al.*, 1996) using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI). The IC_{50} was defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells. In addition, selectivity index (SI) values were determined as the ratio of cytotoxicity over the MIC. The cytotoxicity was determined by exposing the Vero cells to different concentrations of the samples. Stock solutions of the samples were prepared at 12.8 mg/mL^{-1}, the positive control Rifampin (RMP) at 100 mg/mL^{-1} in DMSO. Geometric three-fold dilutions were performed in 96 well clear cell culture plates using the cell culture medium MEM (Gibco, Grand Island, NY) supplemented with 10% of fetal bovine serum (HyClone, Logan, UT). Final DMSO concentrations did not exceed 1% v/v.

Drug dilutions were distributed in duplicate in 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at a volume of 50 μl per well. An equal volume containing 5×10^5 Vero cells (CCL-81; American Type Culture Collection, Rockville, MD) was added to each well and incubated at 37°C in an atmosphere of 5% CO_2 in air. After 72 hours, cell viability was measured using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Absorbance 490 nm was read in a Victor II reader (Perkin Elmer Life Sciences Inc., Boston, MA). The IC_{50} was determined using a Curve-fitting program.

RESULTS AND DISCUSSION

The lipophilic PE and CHCl$_3$ partitions of the total extract of the dried roots of *Angelica sinensis* (Oliv.) Diels contains most of the anti-TB activity of the botanical. In the MABA bioassay, two pathogenic strains of *M. tuberculosis*, H37R*v* and Erdman, were used to evaluate the *in vitro* anti-TB activities of the extracts and the isolated compounds. The results (Table 1) indicate that the methanol crude extract of the roots of *A. sinensis*, together with PE and CHCl$_3$ partitions, showed anti-TB activities with MIC values at 85.1, 64.0 and 63.1 μg/ml, respectively, while the BuOH partitions and the H$_2$O phase of the extract displayed MIC values over 128 μg/ml against the H37R*v* strain. For the Erdman strain, however, only the CHCl$_3$

partition exhibited weak anti-TB activity with an MIC value at 124.6 μg/ml, while the other partitions were considered inactive (MIC values > 128 μg/ml).

Extensive chromatographic fractionation of the combined extract led to the isolation of five polyynes. Their structures were elucidated/identified as falcarindiol (**1**) (Zheng *et. al.*, 1999), 9Z,17-octadecadiene-12,14-diyne-1,11,16-triol,1-acetate (**2**) (Liu *et. al.*, 1998), oplopandiol (**3**) (Kobaisy *et. al.*, 1997), heptadeca-1-ene-9,10-epoxy-4,6-diyne-3,8-diol (**4**) (Fujimoto *et. al.*, 1991), and the new 8-hydroxy-1-methoxy-(*Z*)-9-heptadecene-4,6-diyn-3-one (**5**). As only incomplete spectroscopic data are available in the literature, their ^{1}H and ^{13}C NMR spectral data are summarized for all compounds in Tables 2 and 3. The NMR spectral assignment incorporates results from a full-spin analysis of the ^{1}H spectrum of polyyne **1**, which will provide a template for other polyyne compounds.

Evaluation of the *in vitro* anti-TB activities of the isolates **1-5** indicated that compounds **1** and **2** exhibited the most potent anti-TB activities with MIC values of 6.0 μg/ml and 1.4 μg/ml vs. the Erdman strain, as well as 26.7 μg/ml and 25.3 μg/ml vs. the H37R*v* strain, respectively. Compound **3** displayed moderate activity with MIC values of 49.5 and 50.2 against the two strains, respectively, while the MICs of **4** and **5** were above 60 μg/ml in both strains and, thus, were considered inactive isolates.

Despite the limitations of the dataset, figure 1 provides a preliminary summary of the anti-TB structure-activity relationships of the polyynes, comparing the structures of the isolates **1-5** and sorting them according to their overall *in vitro* anti-TB potency. This comparison reveals that both functional groups in area A (hydroxyl group in position 16 of polyyne **2**, and in position 3 of polyynes **1** and **3**) and B (9,10 olefinic bond) are likely to be essential for the anti-TB activity of polyynes, and is consistent with a recent SAR study reporting a relationship between activity and predicted lipophilicity parameters (Zloh*et. al.*, 2007). Comparing the differences in activity of the pairs **1/3** and **1/4**, the A-terminal unsaturation might contribute to the anti-TB activity, but does not appear to be essential. In addition, the presence of functional groups in area C (an acetate group attached to a saturated aliphatic terminal position) might enhance this activity significantly, as observed in the case of **2**.

Another important biological observation is that, despite the fact that polyynes are widely considered to be cytotoxic, none of the five isolates exhibited measurable cytotoxicity against VERO cells, defining an IC_{50} value of more than 120 μg/ml as a cutoff point. Accordingly, the polyynes **1-3** were found to be rather selective for anti-TB activity, with a selectivity index (SI=IC_{50}/MIC) being as high as >100 for polyyne **2** (precise SI determination limited by the 120 μg/ml cytotoxicity cutoff). Finally, the biological observations summarized in Table 1 represent an interesting reversal in the trend of the activities versus the two tested strains starting out with the extracts: while the crude partitions were more active against H37R*v*, the isolated polyynes exhibited stronger activities against the Erdman strain. Because the anti-TB activity of the extract can be more broadly ascribed to non-polar components, the isolated polyynes represent an important, but not the only part of the active principle. Further research is required to influence the synergy (Inui *et al.*, 2006;Inui *et al.*, 2007) and the presence of other classes of anti-TB active compounds in Dang Gui botanical preparations. At this point, however, the biological potency of polyyne **2** already warrants its further evaluation using *in vivo* models of tuberculosis infection in mice. The findings also underline the role of polyynes as anti-TB active principles in ethnobotanical preparations, and establish an interesting new biological target for *A. sinensis*.

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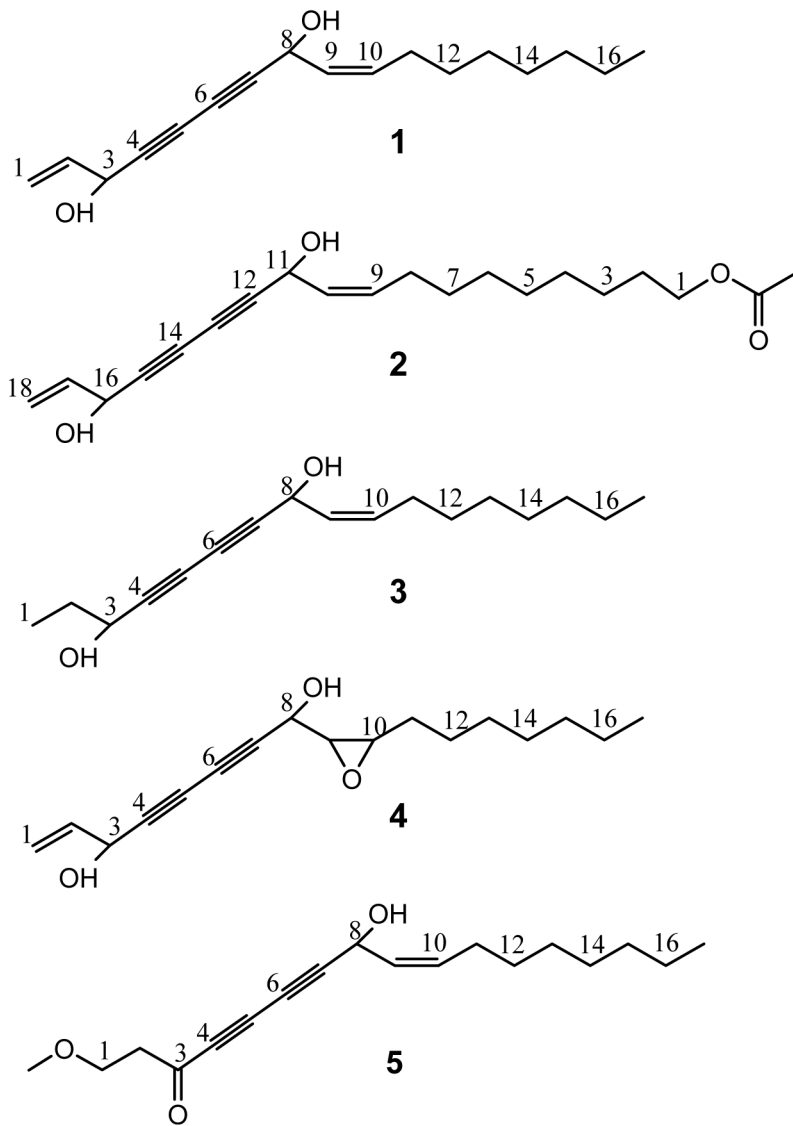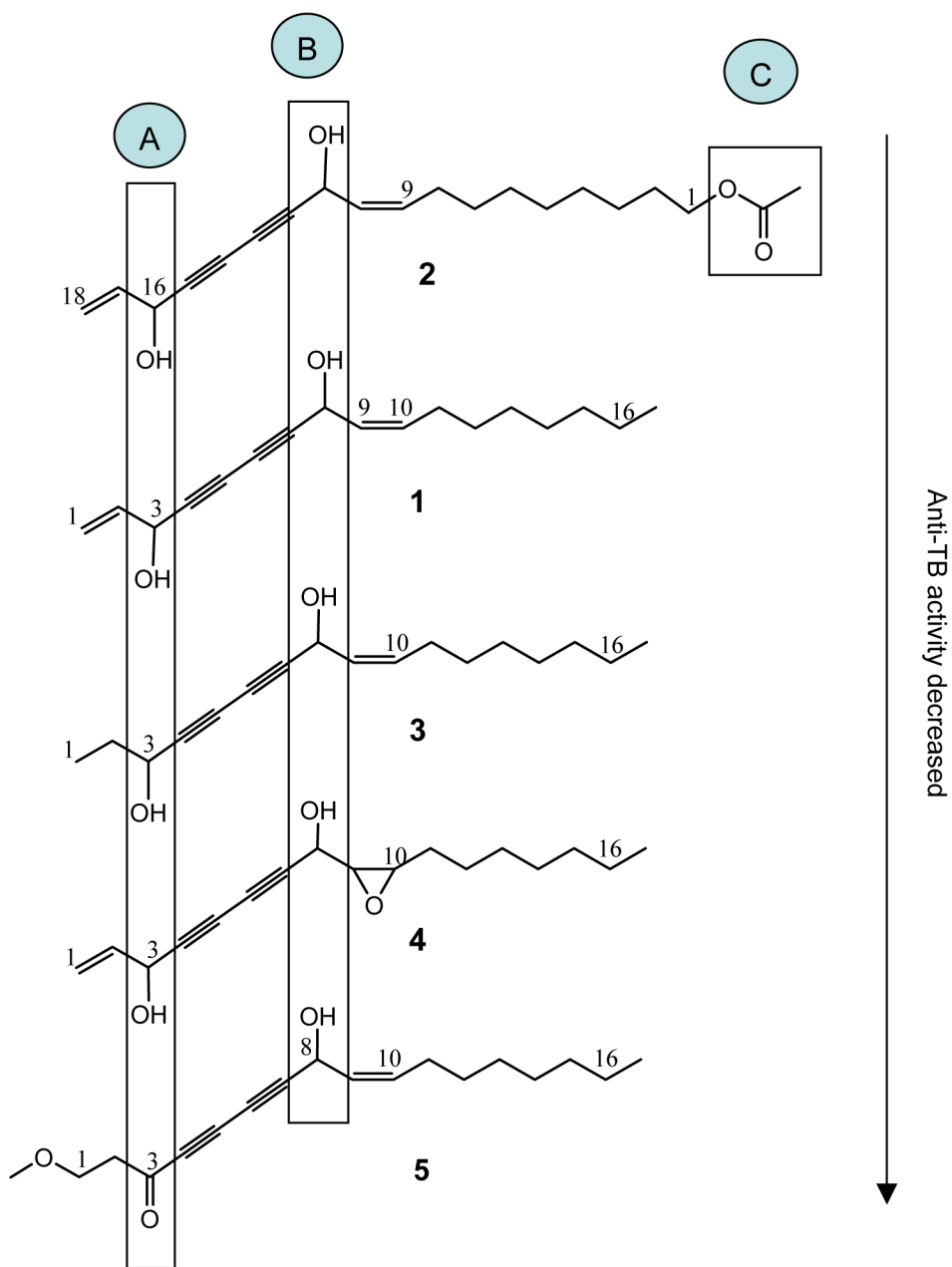
Figure 1.
Preliminary structure-activity relationships of anti-TB polyynes isolated from *A. sinensis*.

Table 1

Anti-TB activities, cytotoxicity (IC_{50}) and selectivity indices (SIs) of extract partitions and the isolated polynes **1-5** from *A. sinensis*

Extract/compounds	MIC,[a][b]		Cytotoxicity IC_{50} (µg/mL)	SI[c]	
	Erdman	H37Rv		Erdman	H37Rv
MeOH extract	>128	85.1			
PE extract	>128	64.0			
CHCl₃ extract	124.6	63.1			
BuOH extract	>128	>128			
H₂O extract	>128	>128			
1	6.0	26.7	>120	>19	>4
2	1.4	25.3	>120	>117	>4
3	49.5	50.2	>120	>2	>2
4	>60	>60	>120	n/a	n/a
5	>60	>60	>120	n/a	n/a
RMP[d]	0.05	0.06	111.6	2232	1860

n/a = not applicable.

[a]Minimum inhibitory concentration defined as the concentration at which the samples exhibit an inhibition of ≥ 90% in the MABA assay.

[b]Average ± SD of triplicate determinations.

[c]Selectivity index (SI = IC_{50}/MIC).

[d]Positive control (Rifampin); negative control was solvent (DMSO).

Table 2

^{1}H-NMR data of polyyne **1-5** from *A. sinensis* [CDCl$_3$, 400 MHz, δ_H (J Hz)]

Position in compounds 1,3-5 [2]	1	2	3	4	5
1 [18]	Ha: 5.466 ddd (17.1, 1.2, 1.0); Hb: 5.254 dt (10.2, 1.2)	Ha: 5.263, dt (1.2, 10.0); Hb: 5.498, dt (1.2, 17.0)	1.017, t (7.4)	Ha: 5.468, d (17.0); Hb: 5.286, d (10.2)	3.719, t (6.0)
2 [17]	5.943, ddd (17.1, 10.2, 5.3)	5.951, ddd (17.0, 10.2, 5.3)	1.749, m	5.954, ddd (17.0, 10.2, 5.2)	2.821, t (6.0)
3 [16]	4.936, ddd (5.3, 1.0, 1.0)	4.952, br d (5.3)	4.385, t (6.4)	4.960, d (5.2)	5.265, dd (8.4, 1.0)
8 [11]	5.213, dt (8.3, 1.0)	5.212, d (8.1)	5.204, d (8.2)	4.296, d (7.4)	5.525, ddt (10.6, 8.4, 1.4)
9 [10]	5.521, dd (10.7, 8.3)	5.526, ddt (10.6, 8.1, 1.0)	5.517, ddt (10.6, 8.2, 1.3)	3.190, dd (7.4, 4.4)	5.670, ddt (10.6, 1.0, 7.6)
10 [9]	5.606, dt (10.7, 7.4)	5.768, ddt (10.6, 7.4, 1.0)	5.613, ddt (10.6, 7.4, 0.9)	3.067, dt (5.5, 4.4)	2.212, ddd (7.6, 7.6, 1.4)
11 [8]	2.105, ddt (8.4, 7.4, 1.2)	2.108, dt (1.3, 7.4)	2.113, ddt (7.4, 7.4, 1.3)	1.560, m	1.329, m
12 [7]	1.390, m	1.391, m	1.389, m	1.215-1.405, m	1.252-1.333, m
13-16 [3-6]	1.240-1.330, m	1.259-1.310, m	1.279-1.293, m	1.215-1.405, m	0.884, t (7.5)
17 [2]	0.882, t (7.0)	1.630, m	0.885, t (7.0)	0.890, t (7.0)	
[1]		4.062, t (6.8)			
[2']		2.051, s			
1-OCH$_3$					3.348, s

Table 3

The ^{13}C-NMR spectral data of polyynes **1-5**, isolated from *A. sinensis* (CDCl$_3$, 100 MHz)

Position compounds 1,3-5 [2]	1	2	3	4	5
1 [18]	117.3	117.3	9.3	117.6	67.1
2 [17]	135.7	135.8	30.7	135.6	45.7
3 [16]	63.4	63.4	64.1	63.3	184.6
4 [15]	78.2	78.4	80.7	76.3	75.7
5 [14]	70.3	70.2	68.6	72.2	77.4
6 [13]	68.7	68.7	68.9	70.7	67.9
7 [12]	79.8	79.7	79.2	78.7	87.6
8 [11]	58.5	58.6	58.7	62.2	59.1
9 [10]	127.6	127.8	127.8	59.5	127.1
10 [9]	134.6	134.4	134.6	57.7	135.8
11 [8]	31.8	29.7	27.7	28.1	28.1
12 [7]	29.3	29.1	29.3	31.7	27.3
13 [6]	29.2	29.1	29.1	29.3	29.4
14 [5]	29.1	28.9	29.2	29.1	29.4
15 [4]	27.7	28.6	22.7	26.6	32.1
16 [3]	22.6	27.6	31.8	22.8	22.9
17 [2]	14.1	25.8	14.1	14.1	14.3
[1]		64.7			
[1']		171.6			
[2']		21.1			
1-OCH$_3$					58.9