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POTENTIAL OF *Azadirachta indica* CELL SUSPENSION CULTURE TO PRODUCE BIOLOGICALLY ACTIVE METABOLITES OF DEHYDROEPIANDROSTERONE

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*Dehydroepiandrosterone (**1**) was investigated for biotransformation studies using the plant cell suspension culture of* Azadirachta indica *A. Juss. for the first time, yielding metabolites **2–6**: 5α,3,17-androstanedione (**2**), 5-androstene-3β,17β-diol (**3**), 3β-hydroxyandrostan-17-one (**4**), 3β,11α-dihydroxy-5-androsten-17-one (**5**), and 3β,7α-dihydroxy-5-androsten-17-one (**6**), whose structures were solved through modern spectroscopic methods. All five compounds **2–6** have not been reported obtained by this way before. This is a new method to biosynthesize compounds **2–6** employing cultured cells of* A. indica*. Metabolites **2**, **3**, and **6** are important biologically active compounds, whereas **4** is a precursor for the production of the 7-hydroxylated compound having antiglucocorticoid and neuroprotective effects.*

Keywords: biotransformation, *Azadirachta indica*, dehydroepiandrosterone, structure elucidation.

Living organism systems, such as microbes (fungi, bacteria, etc.), plant cells and organs, and insects and animals (including cells *in vitro*), are multienzymes systems; therefore, it is possible that a great many products can be obtained from one natural product bioconverted by these systems as an exogenous substrate. The type of biotransformation reactions involves hydroxylation, glycosylation, methylation, acylation, prenylation, sulfation, and many others, and this approach possesses more advantages over chemical reactions by regio- and stereoselectivity, mild conditions, and so on, and there have been many reviews and reports published on it [1–4].

Biotransformation using plant cells and isolated enzymes have immense potential for production of pharmaceuticals. Plant enzyme biocatalysts may be applied to the production of totally new drugs and also may be used to modify existing drugs by improving their bioactivity spectrum. Biological availability of pharmaceuticals can be enhanced by introduction of hydrophilic moieties in the substrate, and the therapeutic action can be prolonged by introduction of protecting groups. Side effects of drugs may be reduced and drug stability may be increased by modification of the parent molecule [5]. The introduction or modification of a functional group into terpenoids and steroids is an important reaction in synthetic organic chemistry. Many studies have been reported on the specific oxidation and reduction of olefines and alicyclic hydrocarbons with chemical reagents [6, 7].

Dehydroepiandrosterone (1), also known as DHEA or prasterone [8], is a naturally occurring and one of the most important hormones produced by the adrenal glands; besides a lot of other advantages, it reduces the risk of heart diseases by lowering cholesterol levels.

In continuation of our previous studies on biotransformation of bioactive compounds using plant cell suspension cultures [9], this report describes the potential of *Azadirachta indica* to produce biologically active compounds by bioconversion of dehydroepiandrosterone (**1**) through using its cell suspension culture, which was being used for the first time for biotransformation studies of this compound, yielding compounds **2–6** (Scheme 1), most of which are biologically active compounds or their precursors. The structures of compounds **2–6** were established with the help of modern spectroscopic techniques and reported data.

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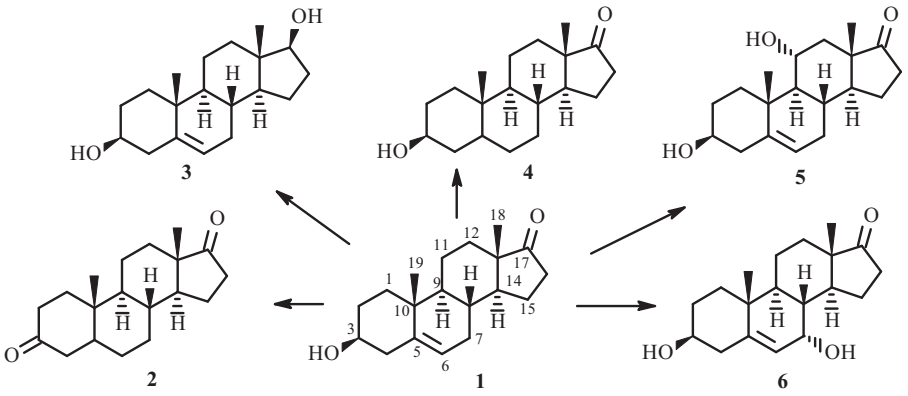
TABLE 1. ^{1}H NMR Chemical Shifts of Compounds **1–6** (400 MHz, $CDCl_3$, δ, ppm, J/Hz)*

C atom	1	2	3	4	5	6
1	1.28 (m)	1.34 (m)	1.32 (m)	1.21 (m)	1.26 (m)	1.31 (m)
	1.82 (m)	2.03 (m)	1.92 (m)	1.79 (m)	2.01 (m)	1.98 (m)
2	1.66 (m)	2.02 (m)	1.58 (m)	1.62 (m)	2.04 (m)	2.03 (m)
	1.93 (m)	2.33 (m)	1.96 (m)	2.08 (m)	2.26 (m)	2.22 (m)
3	3.52 (br.s)	–	3.54 (m)	3.56 (m)	3.53 (m)	3.53(m)
4	2.24 (m)	2.02 (m)	2.22 (m)	2.20 (m)	2.18 (m)	2.21 (m)
	2.34 (m)	2.19 (m)	2.48 (m)	2.41 (m)	2.28 (m)	2.33 (m)
5	–	1.52 (m)	–	1.57 (m)	–	–
6	5.37 (m)	1.33 (m)	5.37 (m)	1.24 (m)	5.36 (m)	5.73 (m)
		1.37 (m)		1.14 (m)		
7	1.20 (m)	1.26 (m)	1.19 (m)	1.28 (m)	1.19 (m)	3.58 (m)
	1.43 (m)	1.31 (m)	1.38 (m)	1.36 (m)	1.41 (m)	
8	1.28 (m)	1.57 (m)	1.51 (m)	1.53 (m)	1.53 (m)	1.67 (m)
9	0.99 (m)	1.76 (m)	0.97 (m)	1.62 (m)	1.93 (m)	1.56 (m)
11	1.62 (m)	1.66 (m)	1.58 (m)	1.56 (m)	3.54 (m)	1.63 (m)
	1.70 (m)	1.72 (m)	1.81 (m)	1.74 (m)		1.77 (m)
12	1.14 (m)	0.97 (m)	1.15 (m)	1.12 (m)	1.58 (m)	1.17 (m)
	1.23 (m)	1.03 (m)	1.22 (m)	1.23 (m)	1.86 (m)	1.26 (m)
14	1.27 (m)	1.15 (m)	1.26 (m)	1.22 (m)	1.23 (m)	1.18 (m)
15	1.90 (m)	1.73 (m)	1.89 (m)	1.83 (m)	1.79 (m)	1.67 (m)
	2.08 (m)	1.94 (m)	2.06 (m)	2.02 (m)	2.03 (m)	1.91 (m)
16	2.06 (m)	1.88 (m)	1.98 (m)	2.16 (m)	1.96 (m)	2.02 (m)
	1.56 (m)	1.58 (m)	1.52 (m)	2.03 (m)	1.79 (m)	1.91 (m)
17	–	–	3.60 (m)	–	–	–
18	1.03 (s)	1.02 (s)	1.04 (s)	0.80 (s)	1.06 (s)	1.01 (s)
19	0.88 (s)	0.87 (s)	0.87 (s)	0.78 (s)	0.88 (s)	0.87 (s)

*Assignment based on COSY and HMQC.



Scheme 1

Biotransformation of dehydroepiandrosterone (**1**) by cell suspension culture of *Azadirachta indica* yielded compounds **2–6**, see Scheme 1.

5α,3,17-Androstanedione (2), $C_{19}H_{28}O_2$, was deduced from HR-EI-MS at *m/z* 288.1012. There were two changes observed upon elucidating the structure on the basis of the spectroscopic data. One was reduction of the olefinic double bond at the C-5 position, and the other was oxidation of the hydroxyl group at C-3, as compared to the substrate **1**. The ^{1}H NMR spectrum of compound **2** displayed no olefinic double bond as well as the peak of hydroxyl, which were present in the ^{1}H NMR spectrum of compound **1** at δ 5.37 and 3.52, respectively.

In the ^{1}H NMR spectrum of compound **2**, two methyl signals were observed at δ 1.02 and 0.87. The ^{13}C NMR spectrum also showed the disappearance of the olefinic double bond in compound **2**. The disappearance of the olefinic double bond was confirmed by ^{13}C NMR spectral data in which additional upfield signals of C-5 methine (δ 46.60) and C-6 methylene (28.62) were seen.

TABLE 2. ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm)*,** Chemical Shifts of DHEA (1) and Its Transformed Products **2–6**

C atom	1	2	3	4	5	6
1	31.47 (t)	38.07 (t)	31.51 (t)	32.14 (t)	31.05 (t)	31.24 (t)
2	31.61 (t)	38.44 (t)	31.64 (t)	36.95 (t)	31.12 (t)	31.12 (t)
3	71.62 (d)	211.57 (s)	71.65 (d)	71.17 (d)	72.32 (d)	70.54 (d)
4	42.23 (t)	44.57 (t)	42.27 (t)	38.08 (t)	41.28 (t)	42.16 (t)
5	141.04 (s)	46.60 (d)	141.68 (s)	44.85 (d)	143.31 (s)	172.60 (s)
6	120.93 (d)	28.62 (t)	120.93 (d)	28.40 (t)	125.52 (d)	123.24 (d)
7	37.20 (t)	31.49 (t)	37.24 (t)	31.52 (t)	20.32 (t)	68.80 (d)
8	31.55 (d)	34.98 (d)	31.57 (d)	35.06 (d)	39.93 (d)	42.66 (d)
9	50.27 (d)	51.20 (d)	51.85 (d)	51.45 (d)	58.21 (d)	51.36 (d)
10	36.65 (s)	35.82 (s)	36.69 (s)	32.04 (s)	36.55 (s)	40.10 (s)
11	20.38 (t)	20.71 (t)	20.41 (t)	20.51 (t)	70.78 (d)	24.38 (t)
12	30.80 (t)	30.54 (t)	30.82 (t)	30.85 (t)	39.93 (t)	30.86 (t)
13	47.54 (s)	47.73 (s)	44.92 (s)	47.80 (s)	47.87 (s)	47.58 (s)
14	51.80 (d)	53.91 (d)	50.33 (d)	54.45 (d)	48.23 (d)	50.01 (d)
15	21.88 (t)	21.78 (t)	21.90 (t)	21.74 (t)	23.99 (t)	21.89 (t)
16	35.83 (t)	35.82 (t)	32.18 (t)	35.79 (t)	36.83 (t)	35.71 (t)
17	221.04 (s)	220.88 (s)	71.20 (d)	221.30 (s)	222.55 (s)	221.6 (s)
18	13.55 (q)	11.47 (q)	12.33 (q)	13.82 (q)	13.46 (q)	13.73 (q)
19	19.42 (q)	13.81 (q)	19.44 (q)	11.18 (q)	18.97 (q)	18.46 (q)

*Multiplicities were determined by DEPT experiments. **Assignment based on HMQC and HMBC.

On the other hand, oxidation of the hydroxyl group at C-3 was also confirmed by ^{13}C NMR spectral data in which an additional downfield signal of the carbonyl group (δ 211.57) and disappearance of downfield methine signals (71.62) at C-3 were observed. Besides these changes, no other change was observed; two methyl signals of C-18 and C-19 were obtained at 11.47 and 13.81, respectively. Thus, compound **2** was identified as 5*α*,3,17-androstanedione [10]. This compound is a strong aromatase inhibitor [11], which is important for the management of breast cancer patients with lesions that are estrogen-receptor positive.

5-Androstene-3*β*,17*β*-diol (3), $C_{19}H_{30}O_2$, was deduced from HR-EI-MS at *m/z* 290.0114. There was an increase in the molecular mass by two units as compared to that of **1**, and a double-dehydration fragment at *m/z* 239 was present. There was only one change observed upon elucidating the structure on the basis of the spectroscopic data. That change was observed at C-17 in which the carbonyl group of substrate **1** was reduced to the hydroxyl group, making the compound more polar. The olefinic double bond at C-5 and the hydroxyl group at C-3 remained intact. The ^1H NMR spectrum of compound **3** displayed an olefinic double bond signal at δ 5.37 similar to compound **1** and two signals of the hydroxyl groups at 3.54 and 3.60. The peak at 3.60 was an extra peak compared to the parent compound **1**.

In the ^1H NMR spectrum of compound **3**, two methyl signals were observed at δ 1.04 and 0.87. The ^{13}C NMR spectrum of compound **3** also showed one extra signal of a hydroxyl group. This was confirmed by ^{13}C NMR spectral data in which an additional downfield signal of C-17 methine (δ 71.20) and disappearance of the C-17 quaternary carbon signal (δ 221.04) were noted. Besides these changes, no other change was observed. The methyl signal of C-18 (12.33) shifted slightly upfield due to the presence of the newly added hydroxyl group, while the methyl signal of C-19 was unchanged. Thus, compound **3** was identified as 5-androstene-3*β*,17*β*-diol. This is another important compound responsible for enhancing resistance to human viral and bacterial diseases by up-regulating host immunity; this molecule is more effective against the discussed diseases than DHEA [12].

3*β*-Hydroxyandrostan-17-one (4), $C_{19}H_{30}O_2$, was deduced from HR-EI-MS at *m/z* 290.2304. The increase in the molecular mass by two units as compared to that of **1** suggested the absence of the oleifinic double bond (in this case) or the reduction of the keto group (as in case of compound **3**). This was confirmed by data obtained through ^1H NMR and ^{13}C NMR. The ^1H NMR did not show any olefinic double bond signal as in the case of the parent compound **1** (δ 5.37) at C-6, and a signal of hydroxyl group was noted at 3.56 along with two methyl signals at 0.80 and 0.78. This study primarily suggests that the olefinic bond has been reduced.

This was then further scrutinized by ^{13}C NMR spectroscopy in which a C-17 quaternary carbon downfield signal (δ 221.30) was speculated. This was confirmed by an additional upfield signals of methine (44.85) at C-5 and methylene (28.40) at C-6. No other change was observed. The methyl signals of C-19 (δ 11.18) was shifted upfield due to the absence of the

olefinic double bond, while the methyl and methine signals of C-18 (13.82) and C-3 (71.17) were unchanged. Thus, compound **4** was identified as 3β-hydroxyandrostan-17-one (epiandrosterone, EpiA), which is a precursor of 7α- and 7β-hydroxylated metabolites in the human brain. These 7-hydroxylated derivatives were shown to exert anti-glucocorticoid and neuroprotective effects [13].

3β,11α-Dihydroxy-5-androsten-17-one (5), $C_{19}H_{28}O_3$, was deduced from HR-EI-MS at *m/z* 304.3312. The increase in the molecular mass by 16 units as compared to substrate **1** indicated the presence of an additional hydroxyl group in the substrate structure because in the mass spectrum all fragments were intact except for an extra peak, which was at *m/z* 304.3312. Then ^{1}H NMR and ^{13}C NMR spectroscopy was performed. The ^{1}H NMR exhibited an olefinic double bond signal as in the case of the parent compound **1** (δ 5.37) at δ 5.36 at the C-6 position, and two signals of the hydroxyl groups were noted at 3.53 and 3.54 along with two methyl signals at 0.88 and 1.06. These values of the ^{1}H NMR signals are the same as in compound **1** except for the value of one hydroxyl group. It is clear that this biotransformed compound has a similar structure as compound **1** but with an extra hydroxyl group.

This was then further seen from the ^{13}C NMR spectrum in which the C-17 quaternary carbon downfield signal (δ 222.55), the downfield signal of the C-3 hydroxyl group at 72.32, the C-5 quaternary carbon signal at 143.31, and the C-6 methylene signal at 125.52 were observed. All these values are comparable to the ^{13}C NMR values of compound **1**, but an additional hydroxyl group signal at C-11 was seen (70.78). The carbon position of this hydroxyl group was assigned from the downfield C-9 methine signal (58.21) and the C-12 (39.93) methylene upfield signal and by correlating the 2D spectral information exhibited in HMQC and HMBC, which were at 50.27 and 30.80, respectively. Apart from this change, no other change was observed. The methyl signals of C-18 (13.46) and C-19 (18.97) were taken into account. Thus, compound **5** was identified as 3β,11α-dihydroxy-5-androsten-17-one [14].

3β,7α-Dihydroxy-5-androsten-17-one (6), $C_{19}H_{28}O_3$, was deduced from HR-EI-MS at *m/z* 304.3204. The increase in the molecular mass by 16 units as compared to substrate (the compound to be biotransformed) **1** indicated the presence of an additional hydroxyl group in the substrate structure because in the mass spectrum all fragments were intact except for an extra peak, which was at *m/z* 304.3204. Then ^{1}H NMR and ^{13}C NMR spectroscopy was performed. The ^{1}H NMR exhibited an olefinic double bond signal as in the case of the parent compound **1** (δ 5.37) at δ 5.73 at the C-6 position, and two signals of the hydroxyl groups were noted at 3.53 and 3.58 along with two methyl signals at 0.87 and 1.01. These values of the ^{1}H NMR signals are the same as in compounds **1** and **5** except for a signal of one hydroxyl group. This again indicates that there is an extra hydroxyl group transferred to DHEA.

This was then further seen from the ^{13}C NMR spectrum in which the C-17 quaternary carbon donwfield signal (δ 221.6), the downfield signal of C-3 hydroxyl group at 70.54, the C-5 quaternary signal at 172.60, and C-6 methine signal at δ 123.24 were observed. All these values are comparable to the ^{13}C NMR values of compound **1**, but an additional hydroxyl group signal at C-7 was seen (δ 68.80). The carbon position of this hydroxyl group was assigned from the C-6 methine (123.24) and C-8 (42.66) methine downfield signals, which were at 120.93 and 31.55, respectively, in substrate **1**. Apart from this change, no other change was observed. The methyl signals of C-18 (13.73) and C-19 (18.46) were taken into account. Thus, compound **6** was identified as 3β,7α-dihydroxy-5-androsten-17-one (7α-OH-DHEA). Some scientists have observed that 7α-OH-DHEA is more active than DHEA in preventing hypoxic cell death of neurons *in vitro*. Therefore, 7-oxygenation seems to be associated with the activation of DHEA [15]. Taking into account the importance of this compound, have recently reported a method for the production of 7α-OH-DHEA using *Mucor racemosus* (A.C.C.C. 0401) [16]. Here we report on the biosynthesis of this bioactive compound **6** using plant cultured cells of *A. indica*, along with other biologically active molecules discussed above.

EXPERIMENTAL

General Methods. The ^{1}H NMR spectra were recorded in $CDCl_3$ on Bruker AM-300 and AM-400 NMR spectrometers with TMS as an internal standard using the UNIX operating system at 300 and 400 MHz, respectively. The ^{13}C NMR spectra were recorded in $CDCl_3$ at 100 MHz on a Bruker AM-400 NMR spectrometer. HR-EI-MS were recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. The IR spectra were recorded on a Jasco A-302 spectrophotometer. The UV spectra were recorded on a Hitachi U-3200 spectrophotometer. The optical rotations were measured on a Jasco DIP-360 digital polarimeter. The melting point was determined on a Buchi 510 apparatus. Column chromatography (CC) was carried out on a silica gel column (70–230 mesh). Purity of the samples was checked by TLC on precoated silica gel GF-254 preparative

plates (20 × 20 cm, 0.25 mm thick, Merck) and were detected under UV light (254 and 366 nm), while ceric sulfate was used as spraying reagent. Dehydroepiandrosterone (**1**) was purchased from Fluka Riedel-deHaen.

Callus Culture. The callus cultures of the plant were derived from young leaves cultivated in 300 mL jars, each containing 25 mL of Murashige and Skoog medium [17] supplemented with sucrose (30 g/L), 3-indolebutyric acid (IBA) (4 mg/L), and 6-benzylaminopurine (BA) (1 mg/L), solidified by agar (6 g/L), and incubated at 25 ± 1°C under complete darkness.

Biotransformation Protocol. Cell suspension cultures were derived from static cultured calli in Erlenmeyer flasks (1000 mL), each containing 400 mL of Murashige and Skoog medium supplemented with the ingredients mentioned above, except for BA and agar. After 15 days of preculturing on a gyratory platform shaker at 100 rpm and with a 16 h photoperiod at 25 ± 1°C, a solution of substrate (100 mg in 1 mL of acetone) was added to each flask through a 0.2 μM membrane filter, and the flasks were placed on a shaker for 5 days. The time course study was performed by taking aliquots from the culture on a daily basis, and the degree of transformation was analyzed by TLC. A negative control containing only plant cell suspension cultures and a positive control containing compound **1** in the medium were also prepared in order to check the presence of plant metabolites in the cell culture and the chemical changes as a result of chemical reaction (if any) due to medium components.

Extraction and Isolation Procedure. After 5 days of incubation, the cells and medium were separated by filtration. The filtrate was extracted with CH_2Cl_2 (3 × 1.5 L), and the cells were extracted in an ultrasonic bath with CH_2Cl_2 (3 × 500 mL) at room temperature. The combined extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure, which afforded a brown residue (1.41 g). The transformed metabolites were isolated from this gummy crude by repeated column chromatography (silica gel) with petroleum ether–EtOAc gradient, affording metabolites **2** (25.6 mg, petroleum ether–EtOAc, 9.1:0.9, R_f 0.43, 7.23% yield), **3** (30.7 mg, petroleum ether–EtOAc, 8.9:1.1, R_f 0.42, 8.67% yield), **4** (28.2 mg, petroleum ether–EtOAc, 8.6:1.4, R_f 0.44, 7.9% yield), **5** (8.51 mg, petroleum ether–EtOAc, 8.2:1.8, R_f 0.40, 2.40% yield), and **6** (11.35 mg, petroleum ether–EtOAc, 8.1:1.9, R_f 0.47, 3.20% yield).

5α,3,17-Androstanedione (2). Colorless solid. EI-MS (*m/z*, I_{rel}, %): 288 [M^+] (100), 270 (26), 255 (38), 244 (79), 217 (85), 199 (10), 167 (7). HR-EI-MS *m/z* 288.1012 (M^+, $C_{19}H_{28}O_2$; calcd 288.1013). ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are given in Tables 1 and 2, respectively.

5-Androstene-3β,17β-diol (3). Colorless solid. EI-MS (*m/z*, I_{rel}, %): 290 [M^+] (100), 270 (55), 255 (91), 244 (8), 217 (11), 199 (19), 165 (16). HR-EI-MS *m/z* 290.0114 (M^+, $C_{19}H_{30}O_2$; calcd 290.0117). ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are given in Tables 1 and 2, respectively.

3β-Hydroxyandrostan-17-one (4). Colorless solid. EI-MS (*m/z*, I_{rel}, %): 290 [M^+] (100), 270 (7), 255 (19), 246 (48), 217 (10), 199 (7), 166 (7). HR-EI-MS *m/z* 290.2304 (M^+, $C_{19}H_{30}O_2$; calcd 290.2306). ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are given in Tables 1 and 2, respectively.

3β,11α-Dihydroxy-5-androsten-17-one (5). Colorless solid. EI-MS (*m/z*, I_{rel}, %): 304.1 [M^+] (17), 286 (100), 270 (11), 233 (4), 215 (2), 199 (2), 161 (10). HR-EI-MS *m/z* 304.3312 (M^+, $C_{19}H_{28}O_3$ calcd 304.3315). ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are given in Tables 1 and 2, respectively.

3β,7α-Dihydroxy-5-androsten-17-one (6). Colorless solid. EI-MS (*m/z*, I_{rel}, %): 304.1 [M^+] (15), 286 (100), 271 (8), 253 (4), 229 (3), 215 (2), 178 (4), 159 (9). HR-EI-MS *m/z* 304.3204 (M^+, $C_{19}H_{28}O_3$ calcd 304.3206). ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are given in Tables 1 and 2, respectively.

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