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Abietane diterpenoids from suspension cultured cells of *Torreya nucifera* var. *radicans*

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Abstract

Three abietane diterpenoids were isolated from the suspension cultured cells of *Torreya nucifera* var. radicans along with four known abietane diterpenoids. Based on spectroscopic evidence, the structures of the three were elucidated as (3*S*,5*R*,10*S*)-7-oxo-12-methoxyabieta-8,11,13-triene-3,11-diol, (3*S*,5*R*,10*S*)-7-oxo-12-methoxyabieta-8,11,13-triene-3,11,14-triol and (5*R*,10*S*)-3-oxo-7*R*,12-dimethoxyabieta-8,11,13-trien-11-ol, respectively. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Torreya nucifera* var. *radicans*; Taxaceae; Gymnospermae; Tissue culture; Diterpenoids; Abietane derivatives

1. Introduction

Abietane diterpenoids are a class of tricyclic diterpenoids, and have been isolated from the leaves of *Torreya nucifera*, Taxaceae, Gymnospermae (Fukushima et al., 1968; Sayama et al., 1971; Harrison and Asakawa, 1987). *T. nucifera* var. *radicans* is a variety of *T. nucifera*, about 2 m high, widely distributed in snowy areas near the sea of Japan in Japan. In our studies of applying plant tissue culture techniques to produce secondary metabolites, we have cultured cells of *T. nucifera* var. *radicans*. We now report the isolation of four known and three new abietane diterpenoids from the cultured cells.

2. Results and discussion

The cell cultures of *T. nucifera* var. *radicans* were induced from young leaves on D2C-NH_4^+ agar medium and maintained on D10CCM-NH_4^+ agar medium. Mass culture was carried out in D10CCM-NH_4^+ liquid medium at ca. 1 kg/cm^2 pressure. After 3 weeks of culture, the cells were harvested. The fresh cells were extracted with MeOH and then the MeOH extract was partitioned between EtOAc and H_2O. The EtOAc extract was applied to a silica gel column and resulting fractions of interest were further purified by C_{18} HPLC to afford three new abietane diterpenoids, compounds **1**, **2** and **3**, along with four known abietane diterpenoids, identified as ferruginol (**4**), hinokiol (**5**), taiwanin F (**6**) and taxusabietane A (**7**) by comparison with the data described in the lit. (Kuo et al., 1985; Harrison and Asakawa, 1987; Yang et al., 1998).

Compound **1** was obtained as pale yellow prisms. Its molecular formula, $C_{21}H_{30}O_4$, was established by high resolution EIMS. The ^{13}C NMR spectrum of **1** (Table 1) revealed the presence of 21 carbon signals, which were sorted by a DEPT experiment into 6 methyls, 3 methylenes, 4 methines and 8 quaternary carbon signals. A methoxyl group and a carbonyl carbon signals were observed at δ 61.1 and 198.7, respectively. The presence of an aromatic ring was suggested from the fully substituted carbon signals appearing at δ 129.5, 140.0, 140.2, 149.4, 151.5, and the methine carbon signal appearing at δ 116.7. The unsaturation degree of 7 and the previously described data above suggested that it was a tricyclic diterpenoid. The ^{1}H NMR spectrum of **1** (Table 2) further suggested that it is a compound with the abietane skeleton: it showed the presence of signals for an isopropyl group at δ 1.25, 1.27 (each 3H, *d*,

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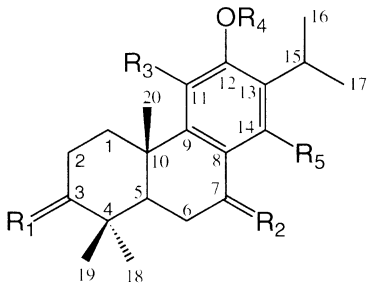

	R_1	R_2	R_3	R_4	R_5
1	OH, H	=O	-OH	$-CH_3$	-H
2	OH, H	=O	-OH	$-CH_3$	-OH
3	=O	H, OCH_3	-OH	$-CH_3$	-H
4	$=H_2$	$=H_2$	-H	-H	-H
5	OH, H	$=H_2$	-H	-H	-H
6	OH, H	=O	-OH	-H	-H
7	=O	=O	-OH	$-CH_3$	-H

$J = 7.0$ Hz) and 3.37 (1H, *qq*, $J = 7.0$, 7.0 Hz) and signals for three methyls on C-4 and C-10 at δ 1.17, 1.26 and 1.63. An aromatic proton signal was also observed at δ 8.07 (1H, *s*). Additionally, a methoxyl group proton signal was observed at δ 3.82. The ^{1}H–^{1}H COSY spectrum showed the existence of–CH_2–CH_2–CH and –CH–CH_2 systems due to positions 1, 2 and 3 of ring A and positions 5 and 6 of ring B, respectively. The structure of **1** was assigned as 7-oxo-12-methoxyabieta-8,11,13-triene-3,11-diol by comparison with the ^{1}H and ^{13}C NMR spectra data (Table 1) of taiwanin F (**6**). In order to confirm this substitution pattern, the HMBC spectrum was measured to give the long-range correlation shown in Fig. 1. Furthermore, the configuration at position 3 was determined to be the *S* configuration according to Mosher's method (Dale and Mosher, 1973; Sullivan et al., 1973). The (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetate (MTPA) ester of **1** showed a downfield shift for H-1 and H-2, and the (*S*)-(−)-MTPA ester of **1** showed a downfield shift for CH_3-18, CH_3-19 and H-5 (Table 3). Therefore, the structure of **1** was determined to be (3*S*,5*R*,10*S*)-7-oxo-12-methoxyabieta-8,11,13-triene-3,11-diol.

Compound **2** was obtained as pale yellow prisms. Its formula was determined to be $C_{21}H_{30}O_5$ from high resolution FABMS. The ^{1}H and ^{13}C NMR spectroscopic data for compound **2** were similar to those of compound **1** except for the aromatic ring. In the ^{13}C NMR spectrum (Table 1), C-8, C-9, C-11 and C-13 showed an upfield shift, whereas C-12 and C-14 showed a downfield shift; in the ^{1}H NMR spectrum (Table 2), the aromatic proton signal due to H-14 was absent. These differences, along with the presence of six quaternary aromatic carbon signals in the DEPT spectrum, indicated that H-14 was substituted in compound **2**. Moreover, the formula of compound **2** contained one more oxygen than that of compound **1**, and a signal for a phenolic hydroxyl proton appeared at δ 14.04, suggesting that H-14 was substituted with a hydroxyl group. In addition, the NOE experiments showed a correlation of the hydroxyl proton (δ 14.04) with the isopropyl group protons, whereas irradiation of the hydroxyl proton signal at δ 10.40 only

Table 1
^{13}C NMR spectroscopic data (δ ppm) for compounds **1** (100 MHz), **2**, **3**, **6** and **7** (75 MHz) in C_5D_5N

Carbon	**6**	**1**	**2**	**7**	**3**
1	35.9	35.5	35.7	36.2	36.0
2	28.7	29.0	28.5	34.9	34.8
3	77.6	77.5	77.4	215.5	217.7
4	39.9	39.9	39.8	47.3	47.0
5	50.7	50.5	49.7	49.8	44.6
6	36.1	36.1	36.2	36.6	25.0
7	198.3	198.7	206.6	197.5	78.4
8	125.5	129.5	113.1	129.1	132.4
9	134.6	140.0	138.3	138.3	132.8
10	40.7	40.9	40.9	39.6	39.0
11	151.0	149.4	141.4	149.5	146.6
12	144.7	151.5	158.1	151.7	149.4
13	140.6	140.2	126.8	140.7	139.5
14	117.9	116.7	155.5	116.4	120.0
15	27.7	27.1	26.1	27.2	27.0
16	23.0[a]	23.7[a]	21.1	23.7[a]	23.9[a]
17	23.2[a]	23.9[a]	21.1	23.8[a]	24.0[a]
18	29.1	28.6	29.1	27.2	28.4
19	16.3	16.3	16.3	20.8	20.8
20	18.6	18.0	17.9	17.9	19.3
12-OCH_3	–	61.1	61.3	61.1	61.1
7-OCH_3	–	–	–	–	56.4

[a] Assignment may be reversed.

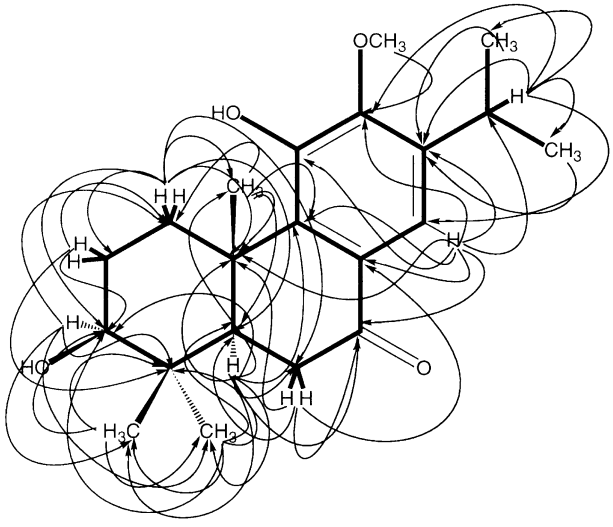

Fig. 1. HMBC correlation of compound **1**.

Table 2
^{1}H NMR spectroscopic data of compounds **1** (C_5D_5N, 400 MHz), **2** (C_5D_5N, 300 MHz) and **3** ($CDCl_3$, 300 MHz)

H	**1**	**2**	**3**
1α	1.69 (*ddd*, 14.0, 14.0, 4.0)[a]	1.68 (*ddd*, 13.5, 13.5, 4.0)	1.97–2.10 (*m*)
β	3.89 (*ddd*, 14.0, 3.5, 4.0)	3.92 (*ddd*, 13.5, 3.5, 3.0)	3.07 (*ddd*, 14.0, 8.5, 6.0)
2α	2.05 (*dddd*, 14.0, 4.5, 4.0, 3.5)	2.00–2.19 (*m*)	2.46 (*ddd*, 15.0, 8.5, 5.5)
β	2.14 (*dddd*, 14.0, 14.0, 11.5, 3.5)		2.70 (*ddd*, 15.0, 10.0, 6.0)
3	3.58 (*dd*, 11.5, 4.5)	3.58 (*dd*, 11.0, 5.0)	–
5	1.96 (*dd*, 13.5, 3.5)	1.80 (*dd*, 13.5, 3.5)	2.51 (*dd*, 13.0, 2.0)
6α	2.91 (*dd*, 17.0, 3.5)	2.77 (*dd*, 17.0, 3.5)	2.06 (*ddd*, 14.0, 2.0, 2.0)
β	2.83 (*dd*, 17.0, 13.5)	2.87 (*dd*, 17.0, 13.5)	1.65 (*ddd*, 14.0, 13.0, 3.0)
7	–	–	4.23 (*dd*, 3.0, 2.0)
14	8.07 (*s*)	–	6.70 (*s*)
15	3.37 (*qq*, 7.0, 7.0)	3.68 (*qq*, 7.0, 7.0)	3.20 (*qq*, 7.0, 7.0)
16	1.25 (*d*, 7.0)[b]	1.56 (*d*, 7.0)[b]	1.24 (*d*, 7.0)
17	1.27 (*d*, 7.0)[b]	1.57 (*d*, 7.0)[b]	1.24 (*d*, 7.0)
18	1.26 (*s*)	1.25 (*s*)	1.18 (*s*)
19	1.17 (*s*)	1.14 (*s*)	1.15 (*s*)
20	1.63 (*s*)	1.58 (*s*)	1.24 (*s*)
12-OCH_3	3.82 (*s*)	3.86 (*s*)	3.74 (*s*)
7-OCH_3	–	–	3.47 (*s*)

[a] δ_H ppm, multiplicity and *J* (Hz) in parentheses.
[b] Assignments may be reversed.

Table 3
^{1}H NMR spectroscopic data for (*R*)-(+)-MTPA and (*S*)-(−)-MTPA esters of compound **1** (400 MHz) in C_5D_5N

	(*S*)-(−)-MTPA ester of **1**	(*R*)-(+)-MTPA ester of **1**	$\delta S-\delta R$
H-1α	1.67 (*ddd*, *J* = 14.0, 14.0, 3.5 Hz)[a]	1.68 (*ddd*, *J* = 14.0, 10.0, 8.0 Hz)	−0.01
H-1β	3.82 (*ddd*, *J* = 14.0, 3.5, 3.5 Hz)	3.85 (*ddd*, *J* = 14.0, 3.5, 3.5 Hz)	−0.03
H-2α	2.00 (*dddd*, *J* = 12.5, 4.5, 3.5, 3.5 Hz)		<-0.02
		2.02–2.08 (*m*)	
H-2β	1.88 (*dddd*, *J* = 14.0, 13.0, 12.0, 3.5 Hz)		<-0.14
H-3α	5.06 (*dd*, *J* = 12.0, 4.5 Hz)	5.06 (*dd*, *J* = 9.5, 7.0 Hz)	0
H-18	1.08 (*s*)	0.96 (*s*)	0.12
H-19	1.01 (*s*)	0.98 (*s*)	0.03
H-5α	2.02 (*dd*, *J* = 12.5, 4.5 Hz)	2.01 (*dd*, *J* = 11.0, 6.0 Hz)	0.01

[a] δ_H ppm, multiplicity and *J* (Hz) in parentheses.

increased the methoxyl proton signal resonance. Therefore, the structure of **2** was (3*S*,5*R*,10*S*)-7-oxo-12-methoxyabieta-8,11,13-triene-3,11,14-triol.

Compound **3** was obtained as colorless needles. Its formula, $C_{22}H_{32}O_4$, was established by high resolution EIMS. Compared with the ^{13}C NMR spectrum of compound **7** (Table 1), there was no signal for the carbonyl group on C-7 of compound **7**. Instead, two new signals, one for a methoxyl group carbon and another for an oxygen-linked methine carbon, appeared at δ 56.4 and δ 78.4, respectively. In addition, in the ^{1}H NMR spectrum, two new signals for a methoxyl group (δ 3.47) and an oxygen-linked methine proton (δ 4.23) were also observed. These data indicated that a methoxyl group, instead of a carbonyl group, existed at C-7 in compound **3**. Comparing the NMR spectroscopic data with that for compound **7**, the structure of **3** was determined to be (5*R*,10*S*)-3-oxo-7,12-dimethoxyabieta-8,11,13-trien-11-ol. Assignments of the two methoxyl proton signals at δ 3.47 and 3.74 were confirmed by a NOE experiment. A NOE correlation was observed for the phenolic hydroxyl proton and isopropyl protons when the methoxyl proton signal at δ 3.74 was irradiated, whereas a NOE correlation was observed for H-5, H-6α and H-7 when the methoxyl proton signal at δ 3.47 was irradiated. Consequently, the signals at δ 3.74 and 3.47 were assigned to the methoxyl groups on C-12 and C-7, respectively. Furthermore, the configuration of C-7 was clarified to be *R* according to the NOE correlation between the methoxyl protons and H-6α.

These three new diterpenoids have a common feature in that all have methoxyl groups on the 7 or (and) the 12 position. Although they could be considered artifacts due to the fact that MeOH was used for the extraction procedure, this possibility was ruled out as they were also isolated from the EtOH extract. In addition, only compounds **1**, **5** and **7** were found in the leaves of intact plants by HPLC analysis (data not shown).

3. Experimental

3.1. General

Mps: uncorr., determined on a Yanagimoto micro melting point apparatus. IR spectra were measured with JASCO IRA-100 spectrometer in KBr. OR were measured with JASCO DIP-370 polarimeter. EIMS and FABMS were measured with JMS-DX300 and JMS-AX505 HA. UV spectra were performed with HITACHI-340 spectrophotometer. NMR spectra were determined on Varian VXR-300 and XL-400 spectrometers. ^{13}C chemical shifts were measured relative to $CDCl_3$ (δ 77.0) or $C5D_5N$–αC (δ 150.0), ^1H chemical shifts were measured relative to chloroform-H (δ 7.26) or pyridine-αH (δ 8.53). HPLC was performed on a Waters model 510 system with 484RI detector, μ BONDASPHERE 5μ C-18 100Å (19×150 mm) column, flow rate 4 ml/min. Column chromatography (CC) was carried out using Wako-gel C-200. TLC was conducted on Kieselgel 60 F254 plates (Merk).

3.2. Induction and subculture of T. nucifera var. radicans cells

Cells were cultured on modified MS solid medium (Murashige and Skoog, 1962) with sucrose 30 g/l, agar 9 g/l, 2,4-dichlorophenoxyacetic acid (2,4-D) 2 mg/l, casamino acid (CA) 0.5 g/l and K^+ instead of NH_4^+ (D2C–NH_4^+) from young leaves of *T. nucifera* var. *radicans*, collected in Tama Forest Science Garden, FFPRI, in June, 1994. The induced cells were subcultured on modified MS solid medium with sucrose 30 g/l, agar 9 g/l, 2,4-D 10 mg/l, CA 1g/l, coconut milk 7% and K^+ instead of NH_4^+ (D10CCM–NH_4^+).

3.3. Mass culture of T. nucifera var. radicans cells

After 4 weeks subculture on D10CCM–NH_4^+ solid medium, cells (22.43 g) were transplanted into two of 1 l flasks containing 250 ml D10CCM–NH_4^+ liquid medium and cultured on a rotary shaker. After 35 days, cells were transplanted into a 5 l jar fermentor containing 3 l D10CCM-NH_4^+ liquid medium and cultured under the pressure of 1 kg/cm^2, 25 °C, 50 rpm.

3.4. Extraction and separation procedures of diterpenoids

After 3 weeks culture, the cells were harvested. The fresh cells (174.2 g) were extracted with MeOH twice at room temp. Evaporation of the solvent under reduced pressure gave the MeOH extract, which was partitioned between EtOAc and H_2O. Removal of the solvent from the EtOAc phase under reduced pressure yielded the EtOAc extract (1.24 g). The EtOAc extract was subjected to silica gel CC and eluted with CH_2Cl_2–MeOH (20:1) to give eight frs. A–H. Fr. B. was subjected to silica gel CC again, with CH_2Cl_2–MeOH (200:1) as eluent to give frs. 1-11.

Further purification of fr. D by HPLC (85% MeOH) afforded hinokiol (**5**) (30.8 mg). Further purification of fr. E by HPLC (80% MeOH) afforded taiwanin F (**6**) (7.1 mg). Further purification of fr. 1, 3, 4 and 7 by HPLC (95% MeOH) afforded ferruginol (**4**) (59 mg), taxusabietane A (**7**) (11 mg), compounds **3** (13.2 mg), **2** (6.7 mg) and **1** (105.7 mg), respectively.

3.5. Compound *1*

Pale yellow prisms. mp. 250–255 °C. $[\alpha]_D$ +15° (MeOH; *c* 0.63). UV (MeOH) λ max nm (log ε): 226 (4.17), 271 (3.96), 314 (3.52). IR (KBr) ν max cm^{-1}: 3450 (OH), 1680 (C=O). EIMS 70 eV, *m/z* (rel. int.): 346 $[M]^+$ (100), 331 $[M–CH_3]^+$ (19), 313 $[M–CH_3–H_2O]^+$ (65), 245 [M-101]$^+$ (88). HR-EIMS *m/z* 346.2128 $[M]^+$, calc. for $[C_{21}H_{30}O_4]^+$, 346.2144.

3.6. Compound *2*

Pale yellow prisms. mp 232–236 °C. $[\alpha]_D$ +53°(MeOH; *c* 0.66). UV (MeOH) λ max nm (log ε): 237 (3.04), 276 (3.04), 366 (2.68). IR (KBr) ν max cm^{-1}: 3425 (OH), 1620 (C=O). EIMS 70 eV, *m/z* (rel. int.): 362 $[M]^+$ (100), 347 [M-CH_3]$^+$ (7). HR–FABMS *m/z* 362.2093 $[M]^+$, calc. for $[C_{21}H_{30}O_5]^+$, 362.2112.

3.7. Compound *3*

Colorless needles. mp. 201–204 °C. $[\alpha]_D$ +116° (MeOH; *c* 0.85). UV (MeOH) λ max nm (log ε): 223 (3.64), 280 (3.32). IR (KBr) ν max cm^{-1}: 3450 (OH), 1700 (C=O). EIMS 70 eV, *m/z* (rel. int.): 360 $[M]^+$ (68), 328 [M-32]$^+$ (47), 243 [M-117]$^+$ (100). HR–EIMS *m/z* 360.2301 $[M]^+$, calc. for $[C_{22}H_{32}O_4]^+$, 360.2286.

3.8. Preparation of (R)-(+)-MTPA and (S)-(−)-MTPA esters of compound *1*

(*R*)-(+)-MTPA/CH_2Cl_2 soln. 1 ml (conc. 46.3 mg/ml) or (*S*)-(−)-MTPA/CH_2Cl_2 soln. 4 ml (conc. 46.3 mg/ml) was added to a mixture of compound **1** (5 mg), *N*,*N*′-dicyclohexylcarbodiimide (47.6 mg for (*R*)-(+)-MTPA; 285.6 mg for (*S*)-(−)-MTPA) and 4-dimethylaminopyridine (5.2 mg), respectively. The reaction proceeded under Ar gas with stirring for 2 h. Then the soln. was filtered and the filtrate was partitioned between CH_2Cl_2 and H_2O. Evaporation of the CH_2Cl_2 phase under reduced pressure gave the CH_2Cl_2 ext. (57.5 mg for (*R*)-(+)-MTPA, 301.2 mg for (*S*)-(−)-MTPA) (Ohtani et al., 1991; Yoshikawa et al., 1996). The CH_2Cl_2 extract was subjected to silica gel chromatography and eluted

with CH_2Cl_2 to give four frs. Further purification of fr. 2 was achieved by repeated HPLC (100% MeOH, and then 90% MeOH) to give (*R*)-(+)-MTPA ester (4.0 mg) and (*S*)-(−)-MTPA ester (4.6 mg).

3.9. (*R*)-(+)-MTPA ester of compound **1**

^{1}H NMR (400 MHz, C_5D_5N): δ 0.96 (3H, *s*, H-18), 0.98 (3H, *s*, H-19), 1.26 (6H, *d*, J=7.0 Hz, H-16, 17), 1.56 (3H, *s*, H-20), 1.68 (1H, *ddd*, J=14.0, 10.0, 8.0 Hz, H-1α), 2.01 (1H, *dd*, J=11.0, 6.0 Hz, H-5α), 2.02–2.08 (2H, *m*, H-2), 2.72–2.83 (2H, *m*, H-6), 3.38 (1H, *qq*, J=7.0, 7.0 Hz, H-15), 3.68 (3H, *s*, -OCH_3), 3.83 (3H, *s*, 12-OCH_3), 3.85 (1H, *ddd*, J=14.0, 3.5, 3.5 Hz, H-1β), 5.06 (1H, *dd*, J=9.5, 7.0 Hz, H-3), 8.05 (1H, *s*, H-14), 7.43–7.54 (3H, *m*, phenyl), 7.83 (2H, *brd*, J=7.5 Hz, phenyl).

3.10. (*S*)-(−)-MTPA ester of compound **1**

^{1}H NMR (400 MHz, C_5D_5N): δ 1.01 (3H, *s*, H-19), 1.08 (3H, *s*, H-18), 1.26 (6H, *d*, J=7.0 Hz, H-16, 17), 1.53 (3H, *s*, H-20), 1.67 (1H, *ddd*, J=14.0, 14.0, 3.5 Hz, H-1α), 1.88 (1H, *dddd*, J=14.0, 13.0, 12.0, 3.5 Hz, H-2β), 2.00 (1H, *dddd*, J=12.5, 4.5, 3.5, 3.5 Hz, H-2α), 2.02 (1H, *dd*, J=12.5, 4.5 Hz, H-5α), 2.78 (1H, *dd*, J=17.0, 12.5 Hz, H-6β), 2.83 (1H, *dd*, J=17.0, 4.5 Hz, H-6α), 3.37 (1H, *qq*, J=7.0, 7.0 Hz, H-15), 3.65 (3H, *s*, -OCH_3), 3.82 (1H, *ddd*, J=14.0, 3.5, 3.5 Hz, H-1β), 3.83 (3H, *s*, 12-OCH_3), 5.06 (1H, *dd*, J=12.0, 4.5 Hz, H-3), 8.04 (1H, *s*, H-14), 7.43–7.54 (3H, *m*, phenyl), 7.83 (2H, *brd*, J= 7.5 Hz, phenyl).

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