A New Cytotoxic Daphnane Diterpenoid, Rediocide G, from *Trigonostemon reidioides*

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Rediocide G (7), a new daphnane diterpenoid, was isolated from the roots of *Trigonostemon reidioides* (Euphorbiaceae), together with two congeners, rediocide A and rediocide B, (+)-syringaresinol, scopoletin, tomentin and stigmasterol. The structure of the new natural product was elucidated by comparison of its NMR and mass spectral data with those of previously known rediocides and confirmed by extensive 2D NMR spectral analysis. Rediocide G (7) was found to be cytotoxic to various cancer cell lines.

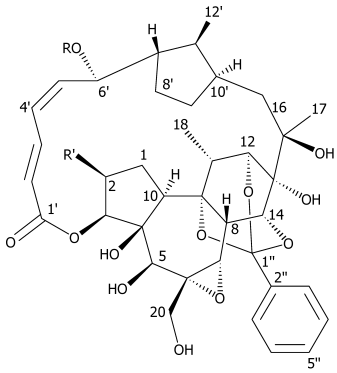
Key words *Trigonostemon reidioides*; Euphorbiaceae; daphnane diterpenoid; rediocide G

During our research for bioactive compounds from Thai Medicinal Plants, we have investigated the *in vitro* cytotoxicity of 39 extracts from 11 Thai plants of 10 families.[1] One of the active extracts from this investigation was the dichloromethane extract of *Trigonostemon reidioides* (KURZ) CRAIB. The plant is known as *Lot thanong* in Thai and belongs to the Euphorbiaceae family. The roots when ground with water have been used in traditional Thai medicine as an emetic for food poisoning, especially from toxic mushrooms and shells, as well as being used as a laxative and antiasthmatic.[2] Previous studies on the chemical constituents of *T. reidioides* have led to the isolation of several classes of compounds such as trigonostemone, a phenanthrenone,[3] afzelechin-(4→8)-afzelechin and lotthanongine, a novel flavonoidal indole alkaloid.[4] In 2000 the first daphnane diterpenoid, rediocide A (**1**), was isolated from the roots of *T. reidioides*.[5] Very recently, five novel daphnane diterpenes, rediocides B—F (**2**—**6**) were isolated from *T. reidioides*[6,7] and these compounds showed very potent anti-flea activity.[6] All rediocides isolated have been found to be among the most potent groups of anti-flea compounds.[6]

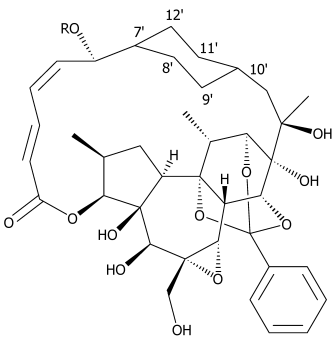
Many daphnane types of diterpenes, known as phorbol esters, have been found in plants of the families Thymelaeceae and Euphorbiaceae. Their bioactivities have indicated a much wider and still largely untapped biological potential. Most daphnanes can produce severe irritant effects, especially on mucous membranes and the eye.[8] These compounds were the first tumor-promoting agents isolated from natural sources, and were known to be powerful activators of protein kinase C.[9] However, some of these diterpenes are not tumor promoters and instead are very effective antiviral (HIV-1) agents.[9] Other biological activity and molecular pharmacology of daphnanes, such as antileukemia, piscicidal, toxicity, anticancer, abortion (birth and fertility regulation) and neurotrophy have been reported.[8] Moreover, some phorbol derivatives, recently isolated from *Sapium indicum* L. (Euphorbiaceae), were found to exhibit antimycobacterial activity.[10]

Results and Discussion

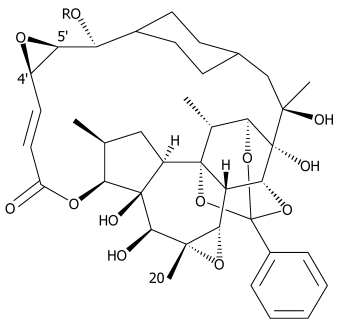
Sequential extraction of the plant material was carried out in *n*-hexane, dichloromethane and 95% ethanol, respectively. The dichloromethane extract was subjected to further purification processes to ultimately give a white powder of three daphnane diterpenoids, rediocide A, rediocide B and the new



(**1**) Rediocide A, R = $COCH_2CHMe_2$, R' = Me
(**3**) Rediocide C, R = COPh, R' = Me
(**5**) Rediocide E, R = $COCH_2CHMe_2$, R' = H
(**6**) Rediocide F, R = COPh, R' = H



(**2**) Rediocide B, R = $COCH_2CHMe_2$
(**7**) Rediocide G, R = COPh



(**4**) Rediocide D, R = COPh

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rediocide G. The HR-FAB-MS (negative) of **7** established a molecular formula of $C_{46}H_{53}O_{13}$ ([M−H]$^-$ *m/z* 813.3484, calculated 813.3486), which was 20 amu more than rediocide A (**1**) and was isomeric with rediocide C (**3**).[6] Comparison of carbon multiplicities in the DEPT spectrum of rediocide A (**1**) and C (**3**) with rediocide G (**7**) revealed that rediocide G (**7**) possesses one less CH_3 and a CH group, and has two additional CH_2 groups, suggesting the presence of a cyclohexyl instead of methylcyclopentyl ring. The COSY spectrum showed the correlations between both H_2-8′ with H_2-9′ and H_2-11′ with H_2-12′. The strong COSY correlations of H-7′ with H-6′, and H-10′ with H_2-16 indicated that the linkage of this cyclohexyl ring is a 1,4-diequatorial configuration at C-7′ and C-10′, while the aromatic region of ^{1}H- and ^{13}C-NMR spectra of rediocide G (**7**) showed the presence of an additional aromatic moiety and the signals for the isobutyl moiety were absent when compared to the NMR spectra of rediocide A (**1**). This indicated that the isobutyl moiety was replaced by the aromatic moiety which was substantiated by the differences in the number of carbons. Some of the stereochemical features of rediocide G (**7**) were obtained from the measurement of *J* couplings and NOESY correlations (Fig. 1, Table 1). The ^{1}H-NMR, COSY, and HMBC spectra re-

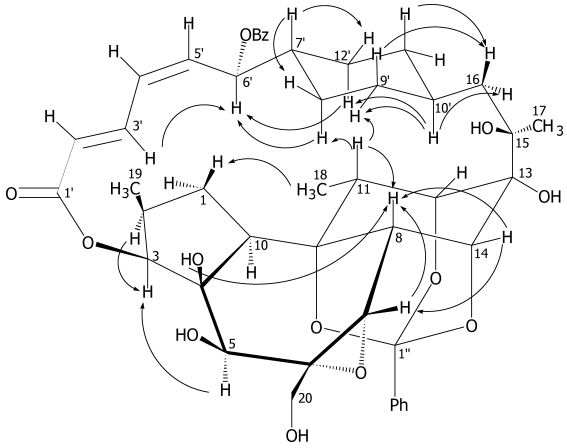

Fig. 1. Significant NOESY Data

Table 1. Rediocide G (**7**) ^{1}H- and ^{13}C-NMR, HMBC, and NOESY Spectral Summary

No.	^{13}C[a]	^{1}H (mult, *J* in Hz)[a]	HMBC[a] (H→C)	NOESY[b]
1	35.3	1.72, m; 2.12, m	H-3, 10, H_3-19	H-2, 3, 10, H_3-18
2	35.4	1.72, m	H-3, 10, H_3-19	H-1, 3, 10
3[b]	80.3	5.15, br d, 3.5	H-1, 5, 10, H_3-19, OH-4	H-1, 2, 5, OH-5
4	80.9	—	H-1, 10, OH-4, OH-5	—
5	70.6	3.78, d, 7.5	—	H-3, 10, OH-5
6	62.0	—	H-5, 7, 8, H_2-20, OH-5	—
7	64.0	3.28, br s	H-5, 8, 14, H_2-20	H-8, H_2-20
8	35.1	4.72, br s	H-7, 11, 10, 14	H-7, 11, 14, OH-4
9	76.9	—	H-7, 8, 10, 11, 12, H_3-18	—
10	46.2	3.10, m	H-1, 3	H-1, 2, 5
11	37.0	3.10, m	H-8, 10, H_3-18	H-8, 8′, 9′
12	84.6	3.54, br d, 1.8	H-14, H_3-18, OH-13	H-16
13	72.0	—	H-12, 14, H_3-17, OH-13, OH-15	—
14	80.1	4.18, br d, 1.6	H-7, 8, 12	H-7, 8
15	76.0	—	H_3-17, OH-13, OH-15	—
16	42.9	1.12, m; 1.80, m	H_3-17, OH-15	H-12, 9′, 10′
17	28.1	1.28, s	OH-15	OH-13
18	19.3	1.49, d, 6.5	H-11, 12	H-1
19	13.4	0.9, d, 6.2	—	—
20	63.2	3.38, m; 3.82, dd, 12.8, 5.7	H-5, OH-20	—
1′	164.8	—	H-3, 2′, 3′	—
2′	124.9	6.04, d, 15.2	H-3′, 4′	H-4′
3′	136.6	7.49, dd, 15.2, 11.1	H-2′, 4′, 5′	H-6′
4′	129.6	6.42, t, 11.1, 11.1	H-2′, 6′	H-2′, 5′
5′	135.3	5.74, dd, 11.1, 9.6	H-3′, 6′	H-4′
6′	78.1	5.49, t, 9.6, 9.6	H-4′	H-3′, 8′, 12′
7′	36.2	1.85, m	H-8′, 12′	H-8′, 12′
8′	29.9	0.90, m; 1.50, m	—	H-6′, 7′, 9′,10′, 12′
9′	31.2	1.12, m; 2.10, m	—	H-11, 8′, 10′
10′	35.7	1.85, m	H-16, 9′	H-16, 8′, 9′, 12′
11′	33.3	0.76, m; 2.20, m	—	H-16, 9′, 12′
12′	32.4	1.12, m; 1.47, m	H-7′	H-6′, 7′, 8′, 9′, 11′
1″	107.6	—	H-12, 14, 3″, 7″	—
2″	139.2	—	H-3″, 4″, 5″, 6″, 7″	—
3″, 7″	125.2	7.59, m	H-4″, 5″, 6″	H-4″, 5″, 6″
4″, 6″	127.5	7.36, m	H-5″	H-3″, 7″
5″	128.7	7.36, m	H-3″, 4″, 6″, 7″	H-3″, 7″
1‴	165.2	—	H-6′, 3‴, 7‴	—
2‴	129.5	—	H-3‴, 4‴, 6‴, 7‴	—
3‴, 7‴	129.1	7.97, dd, 8.5, 1.2	H-4‴, 5‴, 6‴	H-5′, 4‴, 6‴
4‴, 6‴	128.7	7.52, t, 7.5	H-3‴, 7‴	H-3‴, 5‴, 7‴
5‴	133.4	7.65, t, 7.5	H-3‴, 4‴, 6‴, 7‴	H-4‴, 6‴

Hydroxyl groups of rediocide G (**7**) are 4-OH (δ_H, 2.7, s), 5-OH (δ_H, 5.51, d, 7.7), 13-OH (δ_H 4.11, s), 15-OH (δ_H, 4.55, s) and 20-OH (δ_H, 4.46, t, 6.2). *a*) Recorded at 125 MHz for ^{13}C-NMR and 500 MHz for ^{1}H-NMR in DMSO-d_6. *b*) Recorded at 100 MHz for ^{13}C-NMR and 400 MHz for ^{1}H-NMR in $CDCl_3$.

vealed an unusual 12-carbon polyketide to be 2*E*,4*Z*-dodecadienolate, which was confirmed by NOESY correlations. The 2*E* and 4*Z* stereochemistry was evident from the coupling constants of 15.2 Hz and 11.1 Hz for the 2′,3′ and 4′,5′ double bond, respectively. The relative stereochemistry of **7** was deduced using NOESY spectrum. The six-membered ring of daphnane is locked to chair conformation by a 1,3,5-triaxially connected *ortho*-ester group at C-9, C-12, and C-14, which is α-oriented. In the NOESY spectrum of **7**, H-5 was found to exhibit correlations with H-3 and H-10, H-3 to H-2, and H-10 to H-1α thus these protons should be directed to the α-position. The β-oriented proton, H-8, showed a 1,3-diaxial relationship with H-11 and correlation to 4-OH. H-8 appeared as broad singlet due to a 90 degree dihedral angle with the two vicinal protons H-7 and H-14, and to H-11 showing no coupling with H-12, thus placing H-12 and H-14 in the equatorial position. Moreover, H-11 showed a NOESY correlation to H-9′α indicating that the cyclohexyl ring of C-12 polyketide was close and over the cyclohexyl ring of daphnane diterpene. All other observed NOESY correlations are depicted in Fig. 1 and Table 1.

We have also isolated rediocide A[1] (**1**) and rediocide B (**2**) from *T. reidioides*, together with (+) syringaresinol,[11,12] scopoletin,[13] tomentin[14] and stigmasterol. We propose that the methylcyclopentyl ring is the biogenetic precursor of the cyclohexyl ring. The biosynthesis of the cyclohexyl ring presumably passes first through hydroxylation of the methyl group to give the hydroxymethylcyclopentyl ring. Activation of the leaving group *via* phosphorylation of the alcohol, followed by Wagner–Meerwein type rearrangement, can then lead to the cyclohexene ring which can then be converted to the cyclohexyl ring.

We evaluated the cytotoxicity[1] of rediocide G (**7**) against various cell lines and found that the compound exhibited cytotoxicity against GepGII, HeLa, HuCCA-1 and KB cell lines with ED_{50} of 6.4, 4.8, 5.0, and 5.0 μg/ml, respectively. We have also found that rediocide A (**1**) exhibited cytotoxic activity against HeLa (ED_{50} 5.0 μg/ml) and HepG2 (ED_{50} 6.7 μg/ml).[1] The procedure for cytotoxic evaluation was reported in previously published literature.[1]

Experimental

General Procedures Melting points were determined on a melting point apparatus (Buchi 535) and are uncorrected. UV spectra were taken in $CHCl_3$ on a SHIMADZU UV-VIS 2100S spectrometer. IR spectra were recorded in a chloroform solution on a 1760X Perkin-Elmer spectrometer. Mass spectra were measured on Finnigan INCOS 50 and MAT 90. NMR spectra were recorded on Bruker AM 400 at 400 MHz and BRUKER AVANCE 500 at 500 MHz for ^{1}H, and at 100 and 125 MHz for ^{13}C nuclei, respectively, using TMS as an internal standard. HPLC was performed on a Thermo Separation Products system (San Jose, CA, U.S.A.) (pump, P4000; detector, UV6000LP for analysis, UV2000 for preparative mode).

Plant Material The roots of *T. reidioides* were purchased from Loei Province, northern Thailand, in February 2001. Root of *Trigonostemon reidioides* Craib was identified by comparison with the authentic specimen at Forest Herbarium (BKF 36612), National Park, Wildlife and Plant Conservation Department, Ministry of National Resources and Environment, Bangkok, Thailand.

Extraction and Isolation The bioassay-guided fractionation of a dichloromethane extract from the roots of *T. reidioides* using cytotoxicity on KB and HuCCA-1 cell lines as a viability model allowed the isolation of bioactive components from the extracts of plant materials which was carried out with *n*-hexane, dichloromethane and 95% ethanol. Dichloromethane extract (12.3 g) was first submitted to column chromatography on silica gel G (230—400 mesh). The eluent was from 2% dichloromethane in 95% ethanol to pure 95% ethanol to afford 42 mg of the tenth fraction, which was further purified by chromatography on a column of silica gel G (230—400 mesh) to provide the rediocide-containing fractions. Subsequent purification by prep HPLC-ODS with 82% MeOH/H_2O, using a 260 nm UV detector, afforded a white powder consisting of three daphnane diterpenoids, rediocide A (**1**) (14.1 mg), rediocide B (**2**) (1.6 mg) and rediocide G (**7**) (8.3 mg) as a new congener.

Rediocide A (**1**): Colorless solid, mp >230 °C [lit.[5] 213—215 °C, lit.[7] 193—195 °C]; IR ($CHCl_3$) ν_{max}: 3566, 1717, 1690. UV ($CHCl_3$) λ_{max} (log ε): 260 (0.6). HR-FAB-MS (negative) m/z=793.3793 $[M-H]^-$ (Calcd for $C_{46}H_{53}O_{13}$ 793.3799 $[M-H]^-$).

Rediocide B (**2**): White powder, mp >230 °C [lit.[6] pale yellow gum]; IR ($CHCl_3$) ν_{max}: 3749, 1717, 1542, 1508. UV ($CHCl_3$) λ_{max} (log ε): 262 (0.4). HR-FAB-MS (negative) m/z=793.3799 $[M-H]^-$ (Calcd for $C_{46}H_{53}O_{13}$ 793.3799 $[M-H]^-$).

Rediocide G (**7**): White powder, mp >230 °C (decomp.); IR ($CHCl_3$) ν_{max}: 3562, 1717, 1683, 1463, 1334, 1276, 1111, 1072. UV ($CHCl_3$) λ_{max} (log ε): 262 (0.4). ^{1}H-NMR (500 MHz, DMSO-d_6) and ^{13}C-NMR (125 MHz, DMSO-d_6) see Table 1. HR-FAB-MS (negative) m/z=813.3484 $[M-H]^-$ (Calcd for $C_{46}H_{53}O_{13}$ 813.3486 $[M-H]^-$).

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References

1) Tempeam A., Thasana N., Dawornkricharut A., Pavaro C., Ruchirawat S., *Mahidol U. J. Pharm. Sci.*, **29**, 25—31 (2002).
2) Chuakul W., Saralump P., Prathanturarug S., "Medicinal Plants in Thailand," Vol. II, Amarin Printing and Publishing Public Co., Ltd., Bangkok, 1997.
3) Kokpol U., Thebpatiphat S., Boonyaratavej S., Chedchuskulcai V., Ni C. Z., Clardy J., Chaichantipyuth C., Chittawong V., Miles D. H., *J. Nat. Prod.*, **53**, 1148—1151 (1990).
4) Kanchanapoom T., Kasai R., Chumsri P., Kraisintu K., Yamasaki K., *Tetrahedron Lett.*, **43**, 2941—2943 (2002).
5) Jayasuriya H., Zink D. L., Singh S. B., Borris R. P., Nanakorn W., Beck H. T., Balick M. J., Goetz M. A., Slayton L., Gregory L., Zakson-Aiken M., Shoop W., Singh S. B., *J. Am. Chem. Soc.*, **122**, 4998—4999 (2000).
6) Jayasuriya H., Zink D. L., Borris R. P., Nanakorn W., Beck H. T., Balick M. J., Goetz M. A., Gregory L., Shoop W. L., Singh S. B., *J. Nat. Prod.*, **67**, 228—231 (2004).
7) Soonthornchareonnon N., Sakayarojkul M., Isaka M., Mahakittikun V., Chuakul W., Wongsinkongman P., *Chem. Pharm. Bull.*, **53**, 241—243 (2005).
8) He W., Cik M., Appendino G., Van Puyvelde L., Leysen J. E., De Kimpe N., *Mini-Reviews Med. Chem.*, **2**, 185—200 (2002).
9) Pettit G. R., Ducki S., Tan R., Gardella R. S., McMahon J. B., Boyd M. R., Petti G. R., III, Blumberg P. M., Lewin N. E., Doubek D. L., Tackett L. P., Williams M. D., *J. Nat. Prod.*, **65**, 1262—1265 (2002).
10) Chumkaew P., Karalai C., Ponglimanont C., Chantrapromma K., *J. Nat. Prod.*, **66**, 540—543 (2003).
11) Abe F., Yamauchi T., *Phytochemistry*, **27**, 575—577 (1988).
12) Badawl M. M., Handa S. S., Kinghorn A. D., Cordell G. A., Farnsworth N. R., *J. Pharm. Science*, **72**, 1285—1287 (1983).
13) Tsukamoto H., Hisada S., Nishibe S., *Chem. Pharm. Bull.*, **33**, 396—399 (1985).
14) Mengjing C., Linlin H., Guowen Z., *Zhiwu Xuebao*, **30**, 308—311 (1988).