Rediocides B−E, Potent Insecticides from *Trigonostemon reidioides*[⊥]

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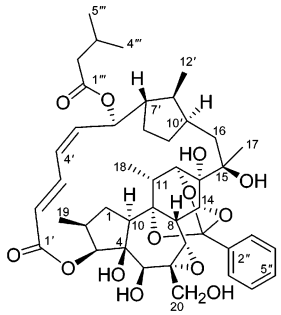
Four new congeners, rediocides B−E (**2**−**5**), of the previously reported rediocide A (**1**) were isolated from a methanol extract of the roots of the plant *Trigonostemon reidioides*. The structures of these minor analogues were elucidated by comparison of their NMR and mass spectral data with those of rediocide A and confirmed by extensive 2D NMR spectral analysis. They all possess potent activity against fleas (*Ctenocephalides felis*) in an artificial membrane feeding system and exhibited LD_{90} values ranging from 0.25 to 0.5 ppm.

Companion animals such as dogs and cats are very frequently infested with the ectoparasitic flea (*Ctenocephalides felis*). This species is the primary contributor to the syndrome "flea allergy dermatitis" in dogs and cats, which is one of the leading causes of veterinary visits in North America and Europe. Flea infestation of owners' homes is also a leading cause of pest control visits and responsible for tons of insecticidal compounds released in the residential environment. Although there have been several new chemical entities (e.g., fipronil, imidacloprid, nitenpyram, selamectin) developed for flea control in the past few years, the massive reproductive capacity of this insect virtually guarantees resistance development against all such compounds. Therefore, for flea control to avoid the fate presently observed in human medicine to antibiotics there needs to be a constant discovery effort to identify new chemical entities. Our objective was to explore and identify orally active anti-flea compounds that represent new chemotypes.

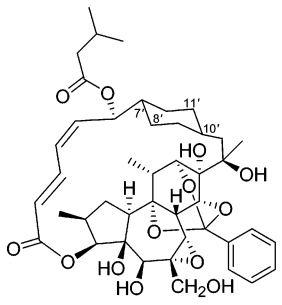
To accomplish our objective we screened natural product extracts of plants and microbial fermentations for insecticidal activity using mosquito larvae[1] (*Aedes aegypti*) as a primary screen followed by a flea membrane assay system[2] as a targeted secondary screen. A methanol extract of the roots of the plant *Trigonostemon reidioides* Craib (Euphorbiaceae) collected in Thailand showed potent activity in this screening program. Bioassay-directed fractionation of this extract led to the discovery of five novel daphnanes, which were named rediocides A−E (**1**−**5**). In 2000, we reported the isolation and structure elucidation of rediocide A (**1**), the major component of the extract.[3] In this paper, we wish to report the isolation, structure elucidation, and the anti-flea activity of four new congeners, rediocides B−E (**2**−**5**).

Silica gel chromatography followed by reversed-phase HPLC of a methylene chloride partition of the original methanolic extract of the roots of *T. reidioides* afforded the new rediocides B−E (**2**−**5**) in a range of 0.006−0.02 wt % of the extract (Experimental Section).

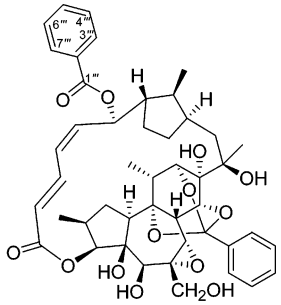
Mass spectral analysis of rediocide B (**2**) indicated a molecular formula of $C_{44}H_{58}O_{13}$ (MW 794), which was isomeric with rediocide A (**1**).[3] Comparison of carbon multiplicities in the DEPT spectrum of the two compounds suggested rediocide B to possess one less CH_3 and a CH group and possess two additional CH_2 groups than rediocide A (Table 1). Comparison of the ^1H and ^{13}C NMR chemical shifts revealed that the H-11′ (δ_C 37.8) and CH_3-12′ (δ_C 16.9) in the cyclopentane ring of the macrocycle of rediocide A were absent in this compound (Table 2). There



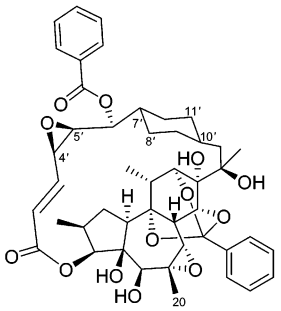
Rediocide A (**1**)



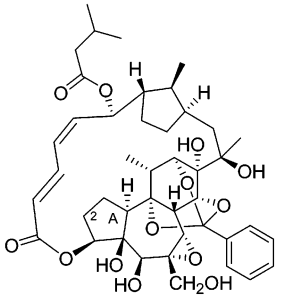
Rediocide B (**2**)



Rediocide C (**3**)



Rediocide D (**4**)



Rediocide E (**5**)

[⊥] Dedicated to the late Dr. Monroe E. Wall and to Dr. Mansukh C. Wani of Research Triangle Institute for their pioneering work on bioactive natural products.

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Table 1. 125 MHz ^{13}C NMR Assignments of Rediocides B−E (**2**−**5**)

position	**2** ($CDCl_3$)	**3** ($CDCl_3$)	**4** (CD_3CN)	**5** ($CDCl_3$)
1	35.7	36.4	37.7	29.3
2	35.7	35.4	36.4	29.0
3	81.6	82.2	83.7	80.7
4	81.3	82.6	82.4	82.1
5	73.2	73.2	74.5	73.1
6	61.0	60.4	60.8	60.4
7	65.2	64.7	69.4	64.1
8	35.7	35.7	36.4	37.6
9	81.3	78.1	78.4	78.0
10	47.4	48.5	47.4	48.7
11	38.1	36.9	38.4	37.6
12	84.8	84.4	86.1	84.5
13	72.3	71.7	73.4	71.6
14	80.4	80.6	81.3	80.7
15	77.7	76.3	77.9	78.0
16	42.9	36.4	43.8	36.4
17	29.9	28.3	28.9	28.3
18	19.8	18.6	20.0	18.5
19	13.2	13.4	13.6	
20	65.5	64.7	23.2	64.6
1′	166.9	169.0	166.7	168.8
2′	123.3	124.8	128.8	124.5
3′	138.4	138.9	141.1	139.1
4′	129.3	130.3	58.2	130.3
5′	136.9	136.6	60.4	136.7
6′	73.2	73.8	80.1	72.9
7′	36.3	51.4	37.7	51.1
8′	30.6	27.3	30.8	27.1
9′	32.0	31.7	31.9	31.6
10′	36.3	40.6	37.0	40.5
11′	34.7	37.7	34.6	37.6
12′	32.7	17.0	33.5	16.8
1	108.7	108.8	108.9	108.8
2	138.6	138.7	139.8	138.7
3	125.1	125.1	126.3	125.1
4	128.1	128.2	129.7	128.1
5	129.3	129.3	129.1	129.3
6	128.2	128.2	129.7	128.1
7	125.1	125.1	126.3	125.1
1‴	172.6	165.7	166.7	172.4
2‴	43.8	133.0	129.6	43.8
3‴	25.8	128.4	130.4	25.9
4‴	22.4	129.6	130.1	22.3
5‴	22.5	130.6	134.3	22.4
6‴		129.6	130.1	
7‴		128.4	130.4	

was no difference in the NMR chemical shifts except for the methylcyclopentyl ring. Hence the methylcyclopentyl ring was replaced by a cyclohexyl ring, which was corroborated by the COSY correlations of H-7′ to both H_2-8′ and H_2-12′. The latter two protons exhibited COSY correlations to H_2-9′ and H_2-11′, respectively. The COSY correlations of H-10′ to H_2-9′ and H_2-11′ helped demonstrate the presence of a closed ring, which was verified by corresponding TOCSY correlations. The COSY correlations of H-7′ to H-6′ and of H-10′ to H_2-16′ established the linkage to the remainder of the molecule. A 1,4-diequatorial configuration at C-7′ and C-10′ was established on the basis of the analogous stereochemistry of rediocide A. Thus, rediocide B (**2**) is a cyclohexyl ring isomer of rediocide A (**1**).

Mass spectral analysis of rediocide C (**3**) provided a molecular formula of $C_{46}H_{54}O_{13}$ (MW 814). The aromatic region of the ^1H NMR spectrum of rediocide C showed the presence of an additional aromatic moiety (Tables 1 and 2). The signals for the isobutyl moiety were absent, indicating its replacement by the aromatic moiety, which was substantiated by the differences in the number of carbons and degrees of unsaturation of the two compounds. This was further supported by the mass spectral data, which indicated a difference of 20 amu, suggesting the replacement of the isobutyrate at C-6′ by a benzoate group. The remaining signals in the ^{13}C and ^1H NMR spectrum of rediocide C (**3**) were identical to that of rediocide A (**1**). Hence rediocide C was deduced to be the C-6′-benzoate analogue of rediocide A (**1**).

Mass spectral analysis of rediocide D (**4**) afforded a molecular formula of $C_{46}H_{54}O_{13}$ (MW 814), which was isomeric with rediocide C (**3**). In addition to the replacement of the methylcyclopentyl ring of macrocycle with a cyclohexyl ring analogous to that seen in rediocide B (**2**), rediocide D (**4**) showed two major differences in the NMR spectra that distinguishes it from the other rediocides (Tables 1 and 2). First, the ^{13}C NMR signals of the C-4′, 5′ double bond (δ_C 130.3 and 136.9) were replaced by signals appearing at δ_C 58.2 and 60.4, respectively. These carbons showed HMQC correlations to protons appearing at δ_H 3.74 (dd, 1H, J = 9.5, 4.5 Hz), and 3.45 (dd, 1H, J = 9.5, 4.5 Hz), respectively. The two pairs of vicinal protons H-3′ and H-4′ as well as H-5′ and H-6′ displayed large couplings (J = 9.5 Hz) indicating *anti*-relationships between H-3′−H-4′ and H-5′−H-6′. By modeling studies[3] the configuration of H-6′ in rediocide A (**1**) has been established as β. By analogy, the stereochemistry of H-6′ was established as β for rediocide D. H-6′ showed an *anti*-relationship with H-5′, indicating that H-5′ has a α-configuration and thus establishing a β-configuration of the C-4−C-5 epoxide. The second change was in the hydroxymethyl group at C-20. The characteristic AB quartet (J = 12 Hz for hydroxymethyl group protons at δ_H 3.77) was absent. Instead there was a new methyl singlet at δ_H 1.36 which showed HMBC correlations to C-5 (δ_C 74.5), C-6 (δ_C 60.8), and C-7 (δ_C 69.3). This allowed the placement of the new CH_3 group at C-6, replacing the hydroxymethyl group. Since rediocide D is isomeric with rediocide C, the oxygenated unit at C-4′, C-5′ of rediocide D (**4**) must be an epoxide instead of a diol, which is consistent with the relative shielding of the ^{13}C chemical shifts of C-4′ and C-5″. Thus, rediocide D (**4**) is a C-4′,5′-epoxy, C-20 deoxy, and C-6′ benzoate congener of rediocide B (**2**).

Mass spectral analysis of rediocide E (**5**) furnished a MW 780, which was 14 amu lower than that of rediocide A (**1**). Comparison of the ^1H and ^{13}C NMR spectra (Tables 1 and 2) of the two rediocides indicated the absence of signals for the C-19 methyl and the C-2 methine in rediocide E and the presence of an extra methylene signal at δ_C 28.99, which was assigned to C-2, which correlated to protons of a methylene at δ_H 1.41. This assignment was confirmed by the COSY and TOCSY correlations of the protons (H-1, -2, -3, and -10) in the cyclopentyl ring A of the diterpenoid. Thus, rediocide E (**5**) is 19-nor-rediocide A (**1**).

Trigonostemone,[4] a phenanthrenone, afzelechin-(4a→8)-afzelechin, and lotthanongine,[5] a flavonoid indole alkaloid,[5] have been reported from *T. reidioides*. The four new congeners (rediocides B−E) (**2**−**5**) reported in this paper extend our discovery of a daphnane diterpenoid from this plant reported previously.[3] The uniqueness of the rediocides is evidenced by the presence of an unusual C-9, C-12, C-14 *ortho*-ester functionality, the unprecedented 12-carbon polyketide extension at C-16, and extremely potent insecticidal activity.

Rediocides B−E (**2**−**5**) were evaluated for their insecticidal properties in an anti-flea artificial membrane feeding assay as detailed earlier.[2] In this assay, rediocides **2**−**5** exhibited LD_{90} values of 0.25, 0.5, 0.25, and 0.5 ppm, respectively, and thus were equipotent with rediocide A

Table 2. 500 MHz ^{1}H NMR Assignments of Rediocides B−E (**2**−**5**)

position	**2** ($CDCl_3$) mult, *J* in Hz	**3** ($CDCl_3$) mult, *J* in Hz	**4** (CD_3CN) mult, *J* in Hz	**5** ($CDCl_3$) mult, *J* in Hz
1	2.1, m	2.1, m	1.8, m	2.41, m
	1.93, m	1.98, m		1.98, m
2	1.73, m	1.73, m	1.70, m	1.41, m
3	4.95, d, 4.6	4.94, d, 4.6	4.84, d, 4.6	5.08, d, 4.0
5	3.86, brs	3.97, brs	3.80, brs	3.90, brs
7	3.42, brs	3.41, brs	3.17, brs	3.35, brs
8	4.63, brs	4.73, brs	4.66, brs	4.68, brs
10	3.11, dd, 13.6, 6.0	2.90, dd, 13.6, 6.0	3.19, dd, 13.6, 6.0	2.74, dd, 13.6, 6.0
11	2.96, q, 6.5	2.96, q, 6.5	3.04, q, 6.5	2.94, q, 6.5
12	3.70, brd, 2.0	3.94, brs	3.62, d, 2.0	3.76, brs
14	4.20, brd, 2.0	4.22, brs	4.22, brs	4.18, brs
16	1.25, m	1.57, m	1.25, m	1.48, m
	2.09, d, 14.8	2.35, d, 16.0	1.80, d, 16	2.29, d, 16
17	1.41, s	1.41, s	1.40, s	1.40, s
18	1.62, d, 6.5	1.62, d, 6.5	1.53, d, 6.5	1.59, d, 6.5
19	1.04, d, 6.8	1.17, d, 6.8	1.06, d, 6.8	
20	3.84, brd, 10	3.80, ABq, 12	1.36, s	3.76, ABq, 12.5
2′	5.90, d, 14.9	5.98, d, 14.9	6.32, d, 15.6	5.89, d, 15
3′	7.50, dd, 14.8, 11.2	7.87, dd, 14.8, 11.2	6.70, dd, 15.6, 9.5	7.70, dd, 16, 12
4′	6.27, t, 11.2	6.27, t, 11.2	3.74, dd, 9.5, 4.5	6.23, t, 11.2
5′	5.60, t, 10.8	5.68, t, 10.8	3.45, dd, 9.5, 4.5	5.56, t, 11.0
6′	5.31, t, 10.8	5.51, t, 10.8	4.87, t, 10.0	5.29, t, 10.0
7′	1.78, m	1.96, m	2.00, m	1.90, m
8′	0.80, m	1.83, m	0.96, m	1.84, m
	1.54, m	1.28, m	1.75, m	1.18, m
9′	0.89, m	1.44, m	1.22, m	1.42, m
	1.56, m	1.64, m	1.90, m	1.63, m
10′	2.16, m	1.65, m	1.92, m	1.65, m
11′	1.20, m	1.75, m	0.88, m	1.75, m
	1.54, m		2.20, m	
12′	1.20, m	0.87, d, 7.2	1.15, m	0.80, d, 7.2
	1.54, m		1.52, m	
3	7.68, dd, 8.0, 1.6	7.74, dd, 8.0, 1.6	7.64, m[a]	7.68, dd, 7.5, 2.0
4	7.38, m	7.38, m	7.38, m[a]	7.38, m[a]
5	7.35, m	7.35, m	7.38, m[a]	7.38, m[a]
6	7.38, m	7.38, m	7.38, m[a]	7.38, m[a]
7	7.68, dd, 8.0, 1.6	7.74, dd, 8.0, 1.6	7.64, m[a]	7.68, dd, 7.5, 2.0
2‴	2.13, d, 7.0			2.15, d, 7.0
3‴	1.98, m	8.05, dd, 7.0, 2.0	8.05, dd, 8.4, 1.2	2.10, m
4‴	0.93, d, 6.4	7.46, t, 8.0	7.54, t, 8.4	0.93, d, 6.4
5‴	0.93, d, 6.4	7.58, brd, 7.5	7.66, m[a]	0.93, d, 6.4
6‴		7.46, t, 8.0	7.54, t, 8.4	
7‴		8.05, dd, 7.0, 2.0	8.05, dd, 8.4, 1.2	
OH-4	2.77, s	3.07, s	2.88	2.94, s
OH-5	3.49, s	3.41, s	3.29, s	3.36, s
OH-13	3.82, s	3.79, s	3.82, s	3.76, s
OH-15				

[a] *J* values could not be determined due to overlap.

(LD_{90} 0.25 ppm).[3] Rediocides A−E (**1**−**5**) constitute one of the most potent groups of anti-flea compounds discovered to date in our research program. In comparison, ivermectin, paraherquamide, and nodulisporic acid A displayed LD_{90} values of 10, >100, and 1 ppm, respectively, in the same assay.[6]

In summary, we have reported four new congeners of rediocide A, highly complex daphnane diterpenoids from *T. reidioides* that are potent anti-flea agents.

Experimental Section

General Experimental Procedures. All reagents were obtained from Sigma-Aldrich and were used without further purification. All solvent extracts were dried on anhydrous Na_2SO_4. Optical rotations were measured on a Perkin-Elmer 241 instrument. The infrared spectra were recorded on a Perkin-Elmer FT instrument. NMR spectra were recorded on Varian Inova 400, 500, or 600 MHz instruments operating at 400, 500, and 600 MHz for ^{1}H and 100, 125, and 150 MHz for ^{13}C nuclei. Mass spectra were recorded on a JEOL SX-102A (electron impact, EI, 90 eV). High-resolution mass spectral analyses were performed either on a Thermo Quest FTMS using electrospray ionization or on a JEOL SX-102A using a FAB probe with perfluorokerosene (PFK) as internal standard. LC-MS was performed on a Thermo Quest LCQ instrument using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). An HP1100 was used for analytical HPLC.

Plant Material. Stems, leaves, and roots of *Trigonostemon reidioides* Craib. (Euphorbiaceae) were collected in Rat Buri Province, Thailand, in December 1988. Voucher specimens (Nanakorn 88213) are deposited in the herbarium of the New York Botanical Garden.

Extraction and Isolation. The roots of *T. reidioides* were extracted with methanol at room temperature. A 20 g portion of the methanol extract was dissolved in 1 L of aqueous 90:10 water−methanol and sequentially extracted with hexane (2 × 500 mL) and methylene chloride (2 × 500 mL). The methylene chloride extract was concentrated to give 4 g of a gum, which was dissolved in methylene chloride−methanol (1:1), adsorbed onto a minimum amount of silica gel, and loaded on a 200 g silica gel column. After eluting the column with 200 mL of 2:1 hexane−acetone the solvent was switched to hexane−acetone (1:1). Rediocides A−E eluted in a broad zone after 250 mL of elution volume with hexane−acetone (1:1) to give 240 mg of enriched fractions. A 60 mg aliquot of

this fraction was purified by HPLC on a Zorbax SB CN column (22.4 × 250 mm) eluting with methanol−water (70:30) at 7 mL/min. The process was repeated three times with the remaining material. The HPLC fractions containing rediocides were concentrated under reduced pressure and lyophilized to afford rediocide B (**2**, 3.8 mg, 0.02%), C (**3**, 3.4 mg, 0.02%), D (**4**, 1.8 mg, 0.01%), and E (**5**, 1.2 mg, 0.006%). The retention times of rediocides A−E (**1**−**5**) on a Zorbax SB CN column (4.6 × 250 mm) eluting with methanol water (3:1) at 1 mL/min were 8.1, 8.6, 9.2, 9.1, and 11.5 min, respectively.

Rediocide B (2): pale yellow gum: $[\alpha]_D^{23}$ +110.8° (*c* 1.75, methanol); IR (ZnSe film) ν_{max} 3458, 2960, 1717, 1688, 1451, 1330, 1268, 1090, 1027 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 208 (11569), 218 (5816), 254 (18085) nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 795.3950 (calcd for $C_{44}H_{58}O_{13}$+H, 795.3956).

Rediocide C (3): pale yellow gum: $[\alpha]_D^{23}$ +130.7° (*c* 2.05, methanol); IR (ZnSe film) ν_{max} 3451, 2957, 1716, 1688, 1451, 1328, 1268, 1095, 1026 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 208 (12210), 234 (17845), 255 (16600) nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 815.3637 (calcd for $C_{46}H_{54}O_{13}$+H, 815.3643).

Rediocide D (4): pale yellow gum: $[\alpha]^{23}_D$ +27.8° (*c* 0.47, methanol); IR (ZnSe film) ν_{max} 3549, 3526, 3464, 2957, 2925, 2854, 1708, 1724, 1635, 1451, 1331, 1268, 1095, 1026 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 208 (15372), 229 (15407), 252 (7071) nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; HREIMS *m*/*z* 814.3564 (calcd for $C_{46}H_{54}O_{13}$, 814.3564).

Rediocide E (5): pale yellow gum: $[\alpha]_D^{23}$ +131.3° (*c* 0.53, methanol); IR (ZnSe film) ν_{max} 3449, 2956, 1689, 1451, 1328, 1268, 1095, 1026 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 208 (15169), 234 (19793), 255 (21458) nm; ^1H and ^{13}C NMR data, see Tables 1 and 2; HREIMS *m*/*z* 780.3720 (calcd for $C_{43}H_{56}O_{13}$, 780.3721).

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