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Algicidal and antifungal compounds from the roots of *Ruta graveolens* and synthesis of their analogs

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Abstract

Bioassay-guided fractionation of the ethyl acetate extract of *Ruta graveolens* roots yielded rutacridone epoxide with potent selective algicidal activity towards the 2-methyl-isoborneol (MIB)-producing blue-green alga *Oscillatoria perornata*, with relatively little effect on the green alga *Selenastrum capricornutum*. The diol-analog of rutacridone epoxide, gravacridondiol, which was also present in the same extract, had significantly less activity towards *O. perornata*. Rutacridone epoxide also showed significantly higher activity than commercial fungicides captan and benomyl in our micro-bioassay against the agriculturally important pathogenic fungi *Colletotrichum fragariae, C. gloeosporioides*, *C. acutatum*, and *Botrytis cineara* and *Fusarium oxysporium.* Rutacridone epoxide is reported as a direct-acting mutagen, precluding its use as an agrochemical. In order to understand the structure–activity relationships and to develop new potential biocides without toxicity and mutagenicity, some analogs containing the (2-methyloxiranyl)-dihydrobenzofuran moiety with an epoxide were synthesized and tested. None of the synthetic analogs showed comparable activities to rutacridone epoxide. The absolute stereochemistry of rutacridone was determined to be 2′(R) and that of rutacridone epoxide to be 2′(R), 3′(R) by CD and NMR analysis.
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Keywords: *Ruta graveolens*; Rutaceae; *Oscillatoria perornata*; *Selenastrum capricornutum*; Algicide; Fungicide; Acridone alkaloids; *Colletotrichum fragariae*; *C. gloeosporioides*; *C. acutatum*; *Botrytis cinearea*; *Fusarium oxysporium*

1. Introduction

Many *Ruta* species are sources of diverse classes of natural products with biological activities including antifungal, phytotoxic, and antidotal activities (Aliotta et al., 1994, 2000; Oliva et al., 2003; De Feo et al., 2002; Sallal and Alkofahi, 1996). Previously, the presence of fungicidal constituents against some agriculturally important fungi in the ethyl acetate extract of *Ruta graveolens* L. leaves has been demonstrated (Oliva et al., 2003). In the present study, we investigated the ethyl acetate extract of the roots of *R. graveolens* for algicidal activity against the 2-methylisoborneol (MIB) producing blue-green alga, *Oscillatoria perornata*, a pest in commercial catfish (*Ictalurus punctatus*) production ponds in the southeastern United States. MIB accumulates in the flesh of catfish, giving them a "musty" off-flavor and thereby rendering them unpalatable. The agents currently approved by United States Environmental Protection Agency to control off-flavor compound-producing blue-green algae, copper-based products and diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea), are limited in their usefulness due to their persistence in the environment, lack of selectivity towards noxious blue-green algae and little margin of safety between phytotoxic and ichthyotoxic concentrations. As an alternative to these synthetic algicides, we are screening natural compounds and extracts from plants to discover selective and environmentally safe algicides for use in catfish aquaculture.

Preliminary bioassays of the ethyl acetate extract indicated the presence of constituents with selective toxicity towards *O. perornata*, when compared to *Selenastrum capricornutum*, a green alga that is found in fish production ponds and considered to be one beneficial type of

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phytoplankton. Bioassay-guided fractionation of this extract led to the isolation of active compounds. These compounds also showed potent fungicidal activity against several agriculturally important pathogenic fungi *Colletotrichum fragariae, C. gloeosporioides*, *C. acutatum*, and *Botrytis cineara* and *Fusarium oxysporium*. This paper describes the isolation, structure elucidation, synthesis, and structure–activity studies of the bioactive constituents from the ethyl acetate extract of *R. graveolens* roots.

2. Results and discussion

The ethyl acetate extract of roots of *R. graveolens* showed moderate toxicity towards *O. perornata* (LCIC = 10 ppm) and at least an order of magnitude greater toxicity compared to *S. capricornutum* (LCIC > 100 ppm). Bioassay-guided fractionation of this extract on silica gel afforded several highly active fractions. Further chromatographic separation of the most active fractions on C-18 silica gel, followed by crystallization, led to the isolation of rutacridone epoxide **(1)** as the most active algicidal compound (lowest-complete inhibition concentration, LCIC = 0.1 ppm, 0.3 μM) (Table 1). The structure of (**1**) was confirmed by direct comparison of NMR and mass spectral data reported in the literature (Nahrstedt et al., 1981).

The IC_{50} of **1** was determined to be 9×10^{-3} μM, which is among the most active natural products against *O. perornata* that we have identified in our algicide-screening program (Fig. 1). The IC_{50} for the green alga *S. capricornutum* was found to be 173×10^{-3} μM, further indicating a high degree of selective toxicity towards *O. perornata*.

The separation of a weakly active column fraction, led to the isolation of another active constituent, which was identified as gravacridondiol (**2**) by analysis of its NMR and mass spectra (Paulini et al., 1991; Bergenthal et al., 1979). The results of the bioassay indicated that **2** was 100× less active than **1** against *O. perornata* (Table 1). Both compound **2** and its possible biosynthetic precursor **1** have been previously isolated from *R. graveolens* (Rozsa et al., 1976; Reisch et al., 1976; Baumert et al., 1987; Nahrstedt et al., 1981, 1985). Rutacridone (**3**), a possible biosynthetic precursor of compound **2**, was isolated from the same extract and had no activity in this bioassay. It has been recently reported that gravacridondiol glucoside (glucoside analog of **2**) as the dominant metabolite and **3** as the major acridone alkaloid of the root tips of *R. graveolens* (Kuzovkina et al., 2004).

Table 1
Algicidal activity of the isolated compounds and synthetic analogs

Test compound	Test organism	
	Oscillatoria perornata LCIC (ppm)	*Selenastrum capricornutum* LCIC (ppm)
(**1**)	0.1	1
(**2**)	10	>100
(**3**)	>100	>100
(**7**)	>100	100
(**8**)	>100	>100
(**9**)	100	100
(**10**)	>100	>100
(**11**)	>100	>100
(**12**)	>100	>100

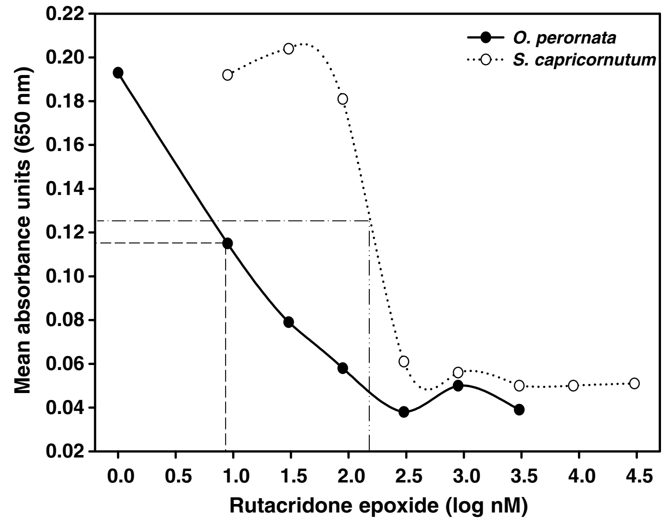


Fig. 1. Rutacridone epoxide (**1**) 96-h IC_{50}. Dotted lines represent IC_{50} intersects of absorbance curves.

These rutacridone analogs were also evaluated for their antifungal activity using bioautography against *C. fragariae*. Compound **1** showed potent antifungal activity at 1 mg/mL, whereas compounds **2** and **3** had no activity against *C. fragariae* at the same concentration. This is in agreement with the observation that rutacridone epoxide (**1**) is a phytoalexin biosynthesized and accumulated from compound **3** in *R. graveolens* suspension cultures after elicitation with dead or live fungi (Eilert et al., 1984; Wolters and Eilert, 1982, 1983; Baumert et al., 1991). LC-MS analysis of the ethyl acetate extract of *R. graveolens* roots indicated that **1** and **3** are present in approximately equal amounts. Compound **1** was further evaluated for fungal growth inhibition (GI) in a micro-bioassay against the plant pathogenic fungi *C. fragariae*, *C. gloeosporioides*, *C. acutatum*, and *Botrytis cinearea*, and *Fusarium oxysporium*. The commercial fungicides captan and benomyl were used as positive controls (Fig. 2). The results of the antifungal micro-bioassay indicated that **1** was significantly more active against *C. fragariae*, *C. gloeosporioides*, and *C. acutatum*, than either captan or benomyl at 2 μM. Rutacridone epoxide (**1**) was the most active compound and demonstrated some selectivity against the three *Colletotrichum* species with an IC_{50} between 0.125 and 1.0 μM. Compound **1** produced 100% GI of *C. fragariae* and *C. gloeosporioides* at 0.5 μM, and was slightly less active against the benomyl resistant species *C. acutatum* (100% GI at 1.0 μM). *Botrytis cinerea* was less sensitive to **1** and, even at the highest concentration, **1** did not cause more that 80% GI. *Fusarium oxysporium* was insensitive to **1** in the concentration range

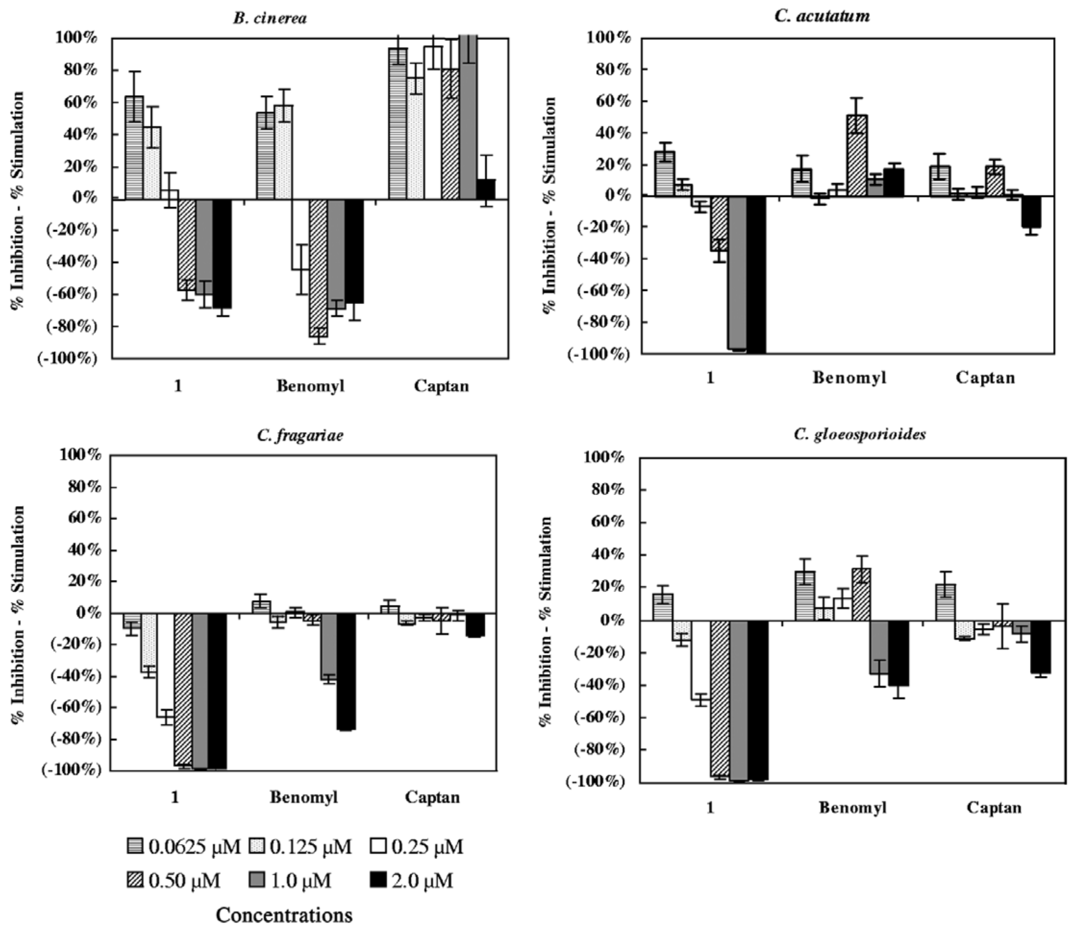


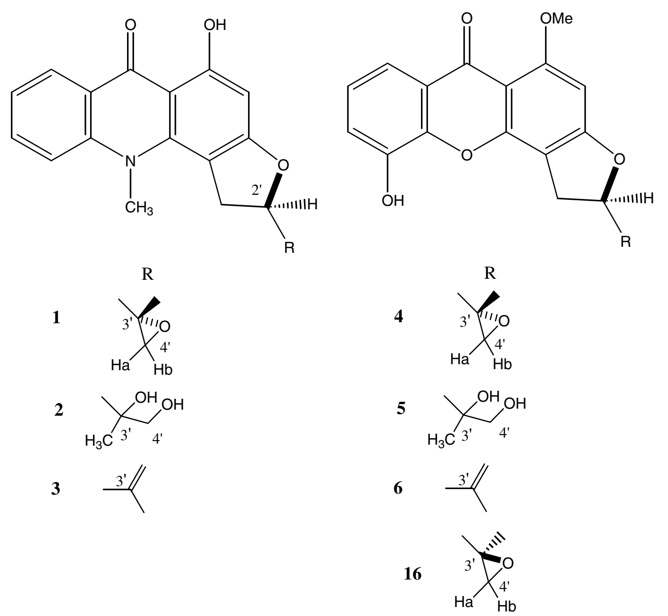
Fig. 2. Fungal growth inhibition of *Botrytis cinerea*, *Colletotrichum acuatum*, *C. fragariae*, and *C. gloeosporioides* at 72 h against rutacridone epoxide and commercial fungicides benomyl, and captan. In vitro threshold values are <3.0 μM for captan and 0.3 μM for benomyl depending on the level of fungicidal sensitivity/resistance profile of the test fungus.

that we tested (data not shown). Due to the high level of antifungal activity of **1** the concentrations evaluated were below the 3.0 μM fungitoxic threshold for benomyl and captan in our assay. In vitro threshold values reported for captan are usually greater than 3.0 μM for (Tabanca et al., 2005) and 0.3 μM for benomyl (Oliva et al., 2003) depending on the level of fungicide sensitivity/resistance profile of the test fungus and the in vitro test system. In fact, both benomyl and captan stimulated fungal growth at some of the low concentrations with some of the fungal species. Such hormetic effects of fungicidal compound at sub-fungitoxic doses are not uncommon (Oliva et al., 2003).

Compound **1** was reported to possess significant direct mutagenic activity in several *Salmonella tryphimurium* strains (Paulini and Schimmer, 1989). We have also shown it to be cytotoxic against several mammalian cell lines (unpublished data). Though the mutagenicity and cytotoxocity of this compound occur at much higher concentrations than those observed for antifungal and algicidal activities, these toxicities will preclude its use as an agrochemical. In the mutagenic assay, compound **3** was found to be mutagenic only after metabolic activation (Paulini and Schimmer, 1989). In the presence of metabolic activation, compound **1** was non-mutagenic apparently due to the formation of compound **2** in the presence of microsomal epoxide hydrolase. The mutagenic activity and antifungal activities of compounds **1** and **2** are parallel. However, compound **2** showed significant algicidal activity indicating that the structural requirements for the mutagenic activity and algicidal activities are different.

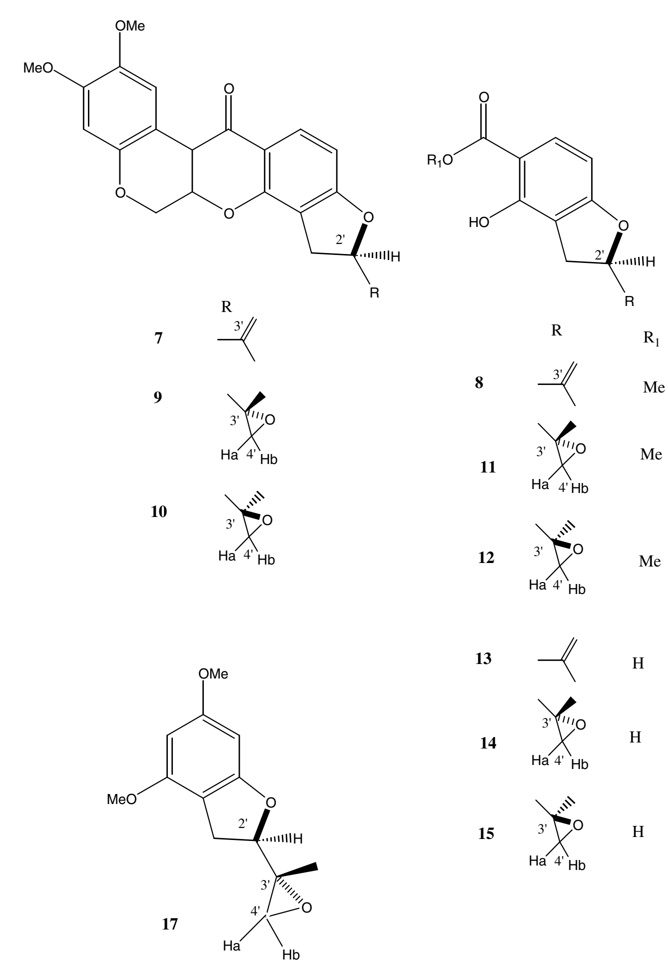
Compound **1** did not show phytotoxicity on higher plants at up to 100 μM. This result was surprising because 0.1 μM of compound **1** completely inhibited growth of the green alga (Fig. 1). These results indicate that the extremely potent algicidal and fungicidal activity of this compound may not be due to general cytotoxicity.

Acridone alkaloids show widespread cytotoxic activity (Kawaii et al., 1999a,b; Su et al., 1992) but only a few xanthones have shown this activity (Peres et al., 2000). Psorospermin (**4**), a natural xanthone with remarkable structural similarities to compound **1** has shown significant cytotoxic activity (Habib et al., 1987). The analogs **5** and **6**, with corresponding structural similarities to **2** (diol) and **3** (double bond) were not cytotoxic (Habib et al., 1987). Therefore, the epoxide on the side chain of furan ring appears to play an important role in the biological activities of these compounds. In order to determine the effect of the epoxide group on the biological activity, several compounds with structural similarities to rutacridone epoxide were prepared and their algicidal and fungicidal activities were evaluated. Rotenone (**7**), a commercially available natural compound with structural similarities to compound **1** and methyl tubaiate (**8**) were converted to their epoxides (**9**,**10**,**11**,**12**) and were tested for algicidal and fungicidal activities along with their parent compounds. None of these compounds showed any activity in these assays.



2.1. Determination of absolute stereochemistry

(−)Rutacridone (**3**) and its epoxide (**1**) have been known for some time. However, the absolute configuration of their stereogenic centers has not yet been determined. In this study we determined the absolute stereochemistry of **3** by comparison of CD spectrum of **3** with that of (−)tubaic acid (**13**), which was prepared from natural rotenone. Both **3** and (−)tubaic acid (**13**) showed negative cotton effects indicating that both have the same absolute stereochemistry, 2′(R) at the chiral center in the dihydrofuran ring. The absolute stereochemistry of the epimeric center of the oxirane ring in the 2′isopropyl) moiety of **1** was determined by comparison of the chemical shift differences [$\Delta\delta(H_a - H_b)$] of the 4′-methylene protons to those of similar compounds with known absolute stereochemistry. The absolute stereochemistry of epimeric epoxides of rotenone has been determined using X-ray crystallography (Habib et al., 1987). Unai and Yamamoto (1973) have observed different chemical shift values for the protons of the methylene signals of the epoxy group in epimeric analogs of rotenone epoxide. The chemical shift difference [$\Delta\delta(H_a - H_b)$] of methylene protons of oxirane ring for 2′(R), 3′(R) analog was 0.25 where as for 2′(R), 3′(S) analogs was 0.11. Similar differences in proton NMR chemical shift values of the methylene protons (4′-H_a and 4′-H_b) of oxirane ring for 2′(R), 3′(R) and 2′(R), 3′(S) could also be observed for tubaic acid epoxide analogs (**14** and **15**) and for related compounds with known absolute stereochemistry such as 2′(R), 3′(R) psorospermin (**4**) and 2′(R), 3′(S) psorospermin (**16**), and synthetic 2′(R), 3′(R) benzofuran analog (**17**). The $\Delta\delta(H_a - H_b)$ values for 2′(R), 3′(R) isomers of rotenone epoxide (Unai and Yamamoto, 1973), tubaic acid epoxide (Habib et al., 1987), psorospermin (Kupchan et al., 1980) and the benzofuran analog (**17**) (Reddy et al., 1987) are 0.28, 0.24, 0.25, and 0.23, respectively, whereas that for 2′(R), 3′(S) isomers of rotenone epoxide (Unai and Yamamoto, 1973), tubaic acid epoxide (Habib et al., 1987), and psorospermin (Ho et al., 1987) are 0.08, 0.11 and 0.07, respectively. The difference of the chemical shifts of H_a and H_b of the methylene protons of the oxirane ring of **1** is 0.27. Therefore, we conclude that the absolute stereochemistry of **1** at the two epimeric centers to be 2′(R), 3′(R).



3. Experimental

3.1. Plant material

Commercially available fresh roots of *R. graveolens* were purchased from Elixir Farm Botanicals, Brixey, MO 65618 USA in May 2002. The plant material was kept at 4 °C until use.

3.2. Extraction

Fresh roots of *R. graveolens* (1.4 kg) were homogenized with EtoAc (4 L) in a commercial blender at ambient temperature and extracted twice for 12 h with EtoAc (8 L). Following filtration through filter paper (Whatman #1), the combined EtoAc extract was concentrated by evaporation under reduced pressure at 40 °C to afford 23.05 g of brown-yellow residue.

3.3. General chemical methods

Extracts were analyzed on 250-μm silica gel TLC plates GF with fluorescent indicator (Analtech, Newark, DE, USA). Iodine vapor, UV light (at 254 and 365 nm), and Dragendorff's and anisaldehyde spray reagents were used for the detection of compounds. Column chromatography was carried out either with kieselgel 60, particle size 0.063–0.2 mm (ICN Biomedicals, Germany), with acetone in hexane in varying amounts or with C-18 silica gel 0.04–0.063 mm particle size (Phenomenex, USA) with MeOH and CH_2Cl_2. All solvents were reagent grade and used without further purification. Rotenone (**3**) and *m*-chloroperbenzoic acid were purchased from Aldrich Chemicals. ^1H and ^{13}C NMR spectra were recorded either on a Bruker AMX NMR spectrometer operating at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR or on a Bruker Avance DPX 300 spectrometer operating at 300 MHz for ^1H NMR and at 75 MHz for ^{13}C NMR. ESIMS was measured using a Bruker Bioapex FTMS with ESI source in positive mode. CD spectra were recorded at 25 °C on Jasco J-715 spectropolarimeter. LC-MS analysis was done on Finnigan AQA Thermoquest in negative mode.

3.4. Bioassay-guided fractionation and isolation of the active constituents

A rapid bioassay for selective algicides was used in order to identify the fractions that are active and selective against *O. perornata* over the green alga *S. capricornutum* according to the published methods (Schrader et al., 1997). The EtoAc extract (20 g) was subjected to silica gel column chromatography, (7 cm id × 40 cm) using hexane (2 L) and gradually increasing acetone by 3%, 5%, 10%, 15%, 20%, 30%, 50%, and 80% in hexane (about 3 L each) up to 100%. Fractions of 350 mL were collected, concentrated at 40 °C, and the fractions with similar TLC profiles were combined to produce 52 fractions. Each fraction was tested in the algicide bioassay.

3.5. Isolation of rutacridone epoxide (*1*)

Fractions 34–38 (122 mg) that showed the highest activity in the algicide assay were further fractionated in a C-18 silica gel column (2.5 cm id × 15 cm) using 5% MeOH in CH_2Cl_2. The active compound was isolated as a bright yellow powder and crystallized from Et_2O in MeOH (86 mg). The identity of this compound was established by comparison of ^1H and ^{13}C NMR spectroscopic data with those reported in the literature (Nahrstedt et al., 1981). CD ($c = 1.6 \times 10^{-4}$, $CHCl_3$): $[\theta]_{265} - 1.7 \times 10^4$, $[\theta]_{275} - 2.8 \times 10^4$, $[\theta]_{328} - 9.4 \times 10^4$.

3.6. Isolation of gravacridondiol (*2*)

Fraction 42 which showed a positive reaction with Dragendorff's reagent was dissolved in MeOH and precipitated with CH_2Cl_2 to obtain a dark yellow powder. This was crystallized from MeOH and CH_2Cl_2 to afford dark yellow crystals (28 mg). The identity of the compound was established by comparison of ^1H and ^{13}C NMR spectroscopic data with those reported in the literature (Paulini et al., 1991; Bergenthal et al., 1979).

3.7. Isolation of rutacridone (*3*)

Fraction 22 was a dark yellow residue, which showed a positive reaction with Dragendorff's reagent, and was dissolved in EtoAc and precipitated with hexane to obtain a yellow powder that was further purified by crystallization to afford yellow crystals (58 mg). The identity of the compound was established by comparison of ^1H and ^{13}C NMR spectroscopic data with those reported in the literature (Paulini et al., 1991; Bergenthal et al., 1979).

CD ($c = 5.8 \times 10^{-5}$, $CHCl_3$): $[\theta]_{268}$ -6.4×10^5, $[\theta]_{275}$ -8.1×10^5, $[\theta]_{330}$ -3.5×10^5.

3.8. Bioassay for algicide activity

Algicidal activities of the compounds were tested in 96-well microplates in a dose–response format according to the previously published methods (Schrader et al., 1997). The lowest-observed effect concentration (LOEC), the lowest-complete inhibition concentration (LCIC), and 50% inhibition concentration (IC_{50}) were determined by graphing the absorbance data.

3.9. Bioassay for fungicide activity against plant pathogenic fungi

Bioautography on silica gel TLC plates was used to detect the presence of antifungal constituents in the extracts, column fractions, and purified compounds and synthesized analogs according to published methods (Wedge et al., 2000; Meepagala et al., 2002; Oliva et al., 2003). In order to evaluate the quantitative fungicidal activity, the active compounds were evaluated in a 6-point dose–response format in the 96-well micro-bioassay using modifications to published methods (Wedge et al., 2000; Meepagala et al., 2002; Oliva et al., 2003). Rutacridone epoxide (**1**) was evaluated at six concentrations (0.0625, 0.125, 0.25, 0.50, 1.0, and 2.0 μM) in comparison to the commercial fungicides captan (multisite inhibitor) and benomyl (β tubulin inhibitor) for activity against *C. fragariae*, *C. gloeosporioides*, *C. acutatum,* and *Botrytis cinerea* and *Fusarium oxysporium*.

3.10. Bioassay for phytotoxic activity

The compounds were evaluated for phytotoxicity on monocots and dicots using lettuce and bentgrass seeds according to published methods (Dayan et al., 2000). Phytotoxic activities were ranked in a scale from 5 to 1 visually with 5 being complete inhibition of germination and 1 being no inhibition.

3.11. Syntheses

3.11.1. Rotenone epoxides (9,10)

Epoxide of rotenone was prepared by adding *m*-chloroperbenzoic acid (17.3 g, 0.1 mol) in 50 mL $CHCl_3$ drop wise into a solution of (**7**) (19.72 g, 0.05 mol) in 100 mL $CHCl_3$ according to the published method (Habib et al., 1987). The product was purified by silica gel column chromatography using EtoAC in hexane to afford a mixture of diastereomeric epoxides as a white powder, which was separated by PTLC using hexane and EtoAC (80:20) as the solvent (5 elusions).

3.11.2. (−)-Tubaic acid (13)

(−)-Tubaic acid was prepared by alkaline degradation of commercially available (−) rotenone with 10% KOH in MeOH as described by Haller and Laforge (1930). The identity was confirmed by comparison of NMR spectroscopic data with those published in the literature (Obara et al., 1976). CD ($c = 2.3 \times 10^{-4}$, $CHCl_3$): $[\theta]_{262} - 9.7 \times 10^3$, $[\theta]_{275} - 1.1 \times 10^4$.

3.11.3. Methyl tubaiate (8)

(−)-Tubaic acid was dissolved in MeOH and treated with ethereal diazomethane at 0 °C. Solvent was removed under vacuum and the product was crystallized from MeOH to give pale yellow crystals (Obara et al., 1976).

3.11.4. Epoxydation of methyl tubaiate

A mixture of methyl tubaiate (100 mg) and *m*-chloroperbenzoic acid (150 mg) in $CHCl_3$ (5 ml) was stirred at room temperature for 24 h. The reaction mixture was diluted with $CHCl_3$ and washed with aqueous sodium carbonate, water dried over magnesium sulfate and evaporated to give a mixture of diastereomeric epoxide. This mixture was separated by preparative TLC using hexanes:EtoAc 90:10 (3 elusions) to give methyl-2′(R),3′(S)-4′-epoxytubaiate (**12**) and methyl-2′(R),3′(R)-4′-epoxytubaiate (**11**).

*3.11.4.1. Methyl 2′(R),3′(S),4-epoxytubaiate (**12**).* White solid m.p. 136–137 °C; $[\alpha]_D^{23}$ −51.2°; HRESI-MS: 234.0894 (calc. for $C_{13}H_{14}O_4$, 234.0892), ^1H NMR δ ($CDCl_3$) 1.43 (3H, *s*, 5′-CH_3), 2.73 and 2.84 (2H, *d*, $J = 4.8$ Hz, 4′-Ha, Hb), 3.09 (1H, *dd*, $J = 16.0$, 8.0 Hz, 1′-H), 3.25 (1H, *dd*, $J = 16.0$, 10.0 Hz, 1′-H), 3.92 (3H, *s*, –OCH_3), 4.83 (1H, *dd*, $J = 10.0$, 8.0 Hz, 2′-H), 6.38 (1H, *d*, $J = 8.4$ Hz, 5-H), 7.70 (1H, *d*, $J = 8.4$ Hz, 4-H).

*3.11.4.2. Methyl 2′(R)-3′(R), 4′-epoxytubaiate (**11**).* White solid m.p. 121–122 °C; $[\alpha]_D^{23}$ −47° ($CHCl_3$; 0.40), HRESI-MS: 234.0893 (calc. for $C_{13}H_{14}O_4$, 234.0892), 1H NMR δ ($CDCl_3$) 1.39 (3H, *s*, 5′-CH_3), 2.70 and 2.93 (2H, *d*, $J = 4.8$ Hz, 4′-Ha, Hb), 3.11 (1H, *dd*, $J = 16.0$, 7.6 Hz, 1′-H), 3.31 (1H, *dd*, $J = 16.0$, 10.0 Hz, 1′-H), 3.91 (3H, *s*, –OCH_3), 4.76 (1H, *dd*, $J = 10.0$, 7.6 Hz, 2′-H), 6.37 (1H, *d*, $J = 8.6$ Hz, 5-H), 7.70 (1H, *d*, $J = 8.6$ Hz, 4-H).

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