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ANTIFUNGAL ACTIVITY OF *TRILLIUM GRANDIFLORUM* CONSTITUENTS

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ABSTRACT.—EtOH extracts of the rhizomes and aboveground portion of *Trillium grandiflorum* showed significant antifungal activity. Bioassay directed fractionation has led to the identification of the active components as the saponin glycosides **1** and **3**.

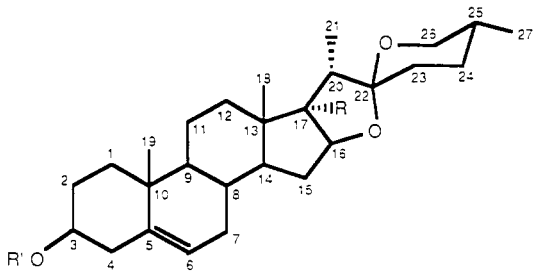
As a part of our continuing search for new anticandidal drugs from natural sources, we have recently reported on the results of liriodenine and its methiodide salt (1). Extracts of the rhizome and aboveground portion of *Trillium grandiflorum* (Michx.) Salisb. (Liliaceae) showed significant in vitro activity against *Candida albicans*. A bioassay-directed fractionation approach resulted in the isolation of the two active components. We herein report the isolation and characterization of these active components and evaluation of their in vitro antifungal and in vivo anticandidal activity.

The rhizome and aboveground portions of *T. grandiflorum* were separately extracted with *n*-hexane (inactive) followed by 95% EtOH. The active EtOH extracts of both the rhizomes and the aboveground portion were partitioned between EtOAc and H_2O. The active H_2O layers were then extracted with *n*-BuOH. The active *n*-BuOH extracts (rhizomes and aboveground) were chromatographed over Si gel G to give two pooled fractions from which two active components, designated TG-I and TG-II, were isolated. TG-I predominates in the rhizomes, and TG-II predominates in the aboveground portion. The isolated yield of TG-I is 0.0094% from the aboveground portion and 0.12% from the rhizomes, while TG-II is 0.113% from the aboveground portion and 0.004% from the rhizomes.

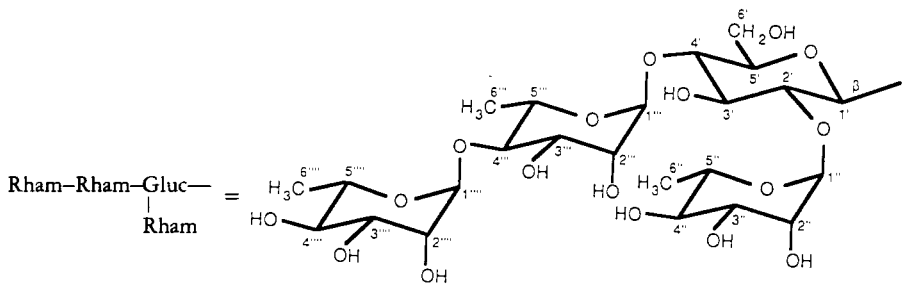
TG-I [**1**] had ^{1}H-nmr and ir data indicative of a complex, polyhydroxylated, natural product. The ^{13}C-nmr (pyridine-d_5) spectrum was particularly informative, because it showed 45 carbon signals that could be attributed to a saponin glycoside containing three sugars (δ C 100.1 d, 101.6 d, and 102.7 d). The aglycone showed signals at δ C 140.8 s, 121.4 d, and 109.1 s characteristic of Δ^5-spirostene type sapogenins (2). Hydrolysis of TG-I produced an aglycone identical with diosgenin [**2**]. The ^{13}C-nmr signals reported for diosgenin and its various glycosides (2) were compared with those of TG-I, thus allowing the remaining signals to be assigned to the three sugars. These data suggested that the three sugars were rhamnose (two) and glucose (one). A review of the literature revealed a report of a saponin glycoside isolated from *Trillium tschonoskii* having this constitution (3). A direct comparison of TG-I [**1**] with dioscin (3) showed the two samples to be identical. The ^{13}C-nmr spectral data were also identical (4).

TG-II [**3**] was established as a saponin glycoside containing the aglycone pennogenin [**4**] and four sugars (three rhamnose and one glucose) on the basis of ^{13}C-nmr spectral data (51 signals). The signals at δ C 89.7 s, 89.9 d, 109.5 s, 121.1 d, and 140.5 s were characteristic for pennogenin. Pennogenin rhamnosyl chacotrioside [**3**] has been previously reported (3,5), and comparison of an authentic sample with TG-II showed them to be identical. Although the ^{13}C-nmr spectral data for **3** has not been reported, the ^{13}C-nmr shift assignments for pennogenin and related glycosides (2) and diosgenin [**2**] plus the same sugar arrangement as in **3** (6) have been reported and are totally consistent with data reported here for TG-II [**3**].

TG-I [**1**] exhibited good inhibition against *C. albicans* B311 in a qualitative agar well-diffusion assay. The MIC of TG-I (determined in yeast nitrogen broth) was found



2 R=R′=H
4 R=OH, R′=H
1 R=H, R′=Rham-Gluc—
Rham
3 R=OH, R′=Rham-Rham-Gluc—
Rham



to be 1.56 μg/ml for *C. albicans* B311, 3.12 μg/ml for *C. albicans* ATCC 10231, and 6.25 μg/ml for *C. albicans* WH. TG-II [**3**] also exhibited good activity against *C. albicans* in the agar well-diffusion assay. The MIC values of TG-II were 6.25 μg/ml for *C. albicans* B311 and 12.5 μg/ml for *C. albicans* ATCC 10231 and WH. By comparison, the MIC value of amphotericin B, the current drug of choice for disseminated candidiasis, ranges from 0.39 μg/ml to 1.56 μg/ml for these strains of *C. albicans*.

Based on these data, both TG-I and TG-II were further evaluated for in vivo efficacy and toxicity. Prior to the initiation of in vivo efficacy studies, a determination of acute toxicity, as median lethal dose (LD_{50}), of each compound was undertaken. The LD_{50} of TG-I was estimated at 38 mg/kg following intraperitoneal (ip) administration and between 10 and 20 mg/kg following intravenous (iv) administration. TG-II exhibited similar acute toxicity with a LD_{50} value of 28.0 mg/kg following ip administration and between 5 and 10 mg/kg following iv administration. The reported LD_{50} for amphotericin B following iv administration is between 1 and 4 mg/kg (7,8).

The in vivo efficacy of each compound was determined in mice infected with a lethal dose of *C. albicans* B311 (via iv injection). No significant ($p<0.06$) reduction in the number of recovered colony forming units (cfu) was observed following a single ip dose (0.1–25 mg/kg) of either TG-I or TG-II. A single dose of amphotericin B (0.5 mg/kg) was found to reduce the number of recovered cfu by 97.5% ($p=0.063$).

It is well documented that saponins similar to **1** and **3** exhibit a variety of biological activities, including antibacterial and antifungal activity (9). Therefore, TG-I [**1**] and TG-II [**3**] were also evaluated for antimicrobial activity against a number of other fungi and bacteria. Both compounds were found to exhibit some in vitro activity against four

other genera of fungi. The MIC values of TG-I and TG-II for the yeasts *Cryptococcus neoformans* and *Saccharomyces cerevisiae* and for the filamentous fungi *Aspergillus flavus, A. fumigatus,* and *Trichophyton mentagrophytes* are summarized in Table 1. Neither compound showed any significant antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* or *Mycobacterium smegmatis*. To our knowledge this is the first report of the antifungal activity and acute toxicity of **1** and **3**.

TABLE 1. Antifungal Activity of TG-I [**1**] and TG-II [**3**].

Organism	MIC (μg/ml)		
	TG-I[**1**]	TG-II [**3**]	Amphotericin B
Candida albicans NIH B311	1.56	6.25	0.39
Candida albicans ATCC 10231	3.12	12.5	1.56
Candida albicans WH-D	6.25	12.5	0.78
Cryptococcus neoformans ATCC 32264	25	12.5	1.56
Saccharomyces cerevisiae ATCC 9763	25	12.5	25
Aspergillus flavus ATCC 9170	100	100	50
Aspergillus flavus ATCC 26934	50	100	100
Trichophyton mentagrophytes ATCC 9972	6.25	3.12	50

EXPERIMENTAL

PLANT MATERIAL.—The entire plant of *T. grandiflorum* was collected in spring 1985, in the southwest corner of Seneca County in Ohio. The rhizomes were separated from the aboveground portion. The plant was verified by Dr. Marvin Roberts, Ohio State University (OSU). Voucher specimens are on deposit at the OSU Herbarium.

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler hot stage instrument and are uncorrected. The ir spectra were obtained on a Perkin-Elmer 281 B spectrometer. ^{13}C-nmr were all recorded in pyridine-d_5 on a Varian VXR-300 spectrometer operating at 75.1 MHz for ^{13}C. The multiplicities were confirmed by the APT and DEPT experiments. Abbreviations s,d,t,q refer to quarternary, methine, methylene, and methyl carbon signals, respectively. Tlc was performed on Si gel (Brinkmann Instruments, Sil-G UV_{254}) using $CHCl_3$-MeOH-H_2O (7:3:0.5) as eluent and anisaldehyde-H_2SO_4 spray reagent for visualization.

QUALITATIVE ANTIMICROBIAL EVALUATION.—In vitro evaluation of anticandidal activity was accomplished using the agar-well diffusion assay previously described (10,11) with the following modifications. *C. albicans* NIH B311 (used to induce experimental disseminated candidiasis) was used for the initial qualitative evaluation of anticandidal activity. The organism was grown in Sabouraud-dextrose broth (SDB) for 24 h at 37°, at which time the cells were harvested by centrifugation (4°, 2000 rpm, 3 min). After centrifugation, the cells were washed and suspended in sterile 0.9% saline to give a final concentration of 10^6 cfu/ml (adjusted using a hemocytometer). Culture plates (15 × 100 mm) for the qualitative assay were prepared from 25 ml of Sabouraud-dextrose agar (SDA). Using sterile cotton swabs, the plates were streaked with the suspension (10^6 cfu/ml) of *C. albicans* B311. Cylindrical plugs were removed from the agar plates by means of a sterile cork borer to produce wells with a diameter of approximately 11 mm. To the well was added 100 μl of solution or suspension of an extract, fraction, or pure compound. Crude extracts and fractions were tested at a concentration of 20 mg/ml, whereas pure compounds were tested at 1 mg/ml. When solvents other than H_2O, EtOH, MeOH, DMSO, DMF, or Me_2CO were required to dissolve extracts or compounds, solvent blanks were included. Anticandidal activity was recorded as the width of zone (in mm) from the edge of the agar well to the edge of the zone after incubation of the plates at 37° for 24 h. The antifungal agent amphotericin B was included as positive control in each assay.

In vitro evaluation of antimicrobial activity against other fungi and bacteria was accomplished as previously described (10,11). Test organisms included *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633), *E. coli* (ATCC 10536), *P. aeruginosa* (ATCC 15442), *M. smegmatis* (ATCC 607), *C. neoformans* (ATCC 32264), *S. cerevisiae* (ATCC 9763), *A. flavus* (ATCC 9170), *A. fumigatus* (ATCC 26934), and *T. mentagrophytes* (ATCC 9972).

QUANTITATIVE ANTIFUNGAL EVALUATION.—The MIC of TG-I and TG-II for *C. albicans, C. neo-*

formans, *S. cerevisiae*, *A. flavus*, *A. fumigatus*, and *T. mentagrophytes* were determined using the twofold serial broth dilution assay (10,11). Both compounds were initially tested using a concentration of 100 μg/ml in the first tube. The test compound was added to sterile culture broth (yeast nitrogen broth for *C. albicans*; SDB for others) as a solution in DMSO. The yeast inocula for the MIC determinations were prepared as described for qualitative evaluation. The inocula of filamentous fungi were prepared by the addition of 1 ml of sterile H_2O to stock slant cultures. Using a calibrated sterile wire loop, 10 μl of suspension was used as inoculum for each tube. The MIC value was taken as the lowest concentration of compound that inhibited the growth of the test organisms after 24 h (*C. albicans*) or 48 h of incubation. *C. albicans* cultures were incubated at 37°, while all others were incubated at 30°. The antifungal agent amphotericin B was included as positive control in each assay.

EXTRACTION AND FRACTIONATION.—The aboveground portion (1.18 kg) and the rhizomes (0.75 kg) were percolated (separately) with *n*-hexane to give an *n*-hexane extract (aboveground = 45.0 g; rhizomes = 45.0 g). The plant material was then air dried and percolated with 95% EtOH (20 liters) to give the EtOH extract (aboveground = 190.2 g; rhizomes = 180.0 g). The EtOH extract was then partitioned between 2 liters each of EtOAc and H_2O (3 × 2 liters EtOAc, aboveground = 10.9 g; rhizomes = 6.7 g). The H_2O layer was further extracted with *n*-BuOH (saturated with H_2O) (3 × 650 ml) and then freeze-dried (H_2O, aboveground = 134.5 g; rhizomes = 105.0 g). The combined *n*-BuOH layers were evaporated to dryness (aboveground = 30.5 g; rhizomes = 48.3). Each fraction was subjected to bioassay against *C. albicans*. The activity was found in the EtOH extract and the *n*-BuOH fractions. The final H_2O (freeze-dried) fraction was inactive. Some activity was noted in the EtOAc fraction.

CHROMATOGRAPHY OF THE ACTIVE *n*-BuOH FRACTION.—(a) The 30.5 g of *n*-BuOH fraction (aboveground) was dissolved in 50 ml of warm MeOH. To this warm solution was added (with stirring) a total of 850 ml of Me_2CO. The white precipitate (active) was collected by filtration (14.7 g) and the filtrate evaporated to give a residue (13.0 g). The 14.7 g of white precipitate was chromatographed over Si gel (300 g, 70–270 mesh, 5 cm × 36 cm) using $CHCl_3$-MeOH-H_2O (7:3:0.5) as the eluting solvent. Fractions (125 ml) were combined mainly on the basis of tlc patterns. Fractions 5 and 7 (2.2 g and 1.27 g, two main components) were active and were further chromatographed on Si gel several times (as above) to give 110 mg of TG-I and 1.33 g of TG-II, which were both active.

(b) Chromatography of 46.9 g of *n*-BuOH fraction (rhizomes) on a Si gel column (900 g, 70–270 mesh, 6.8 × 56 cm) using CH_2Cl_2-MeOH-H_2O (8:4:0.1) gave a total of six pooled fractions. Fraction 3 gave 212 mg of TG-I. An additional 703 mg of TG-I was obtained from fraction 4 and the mother liquors of fraction 3. A total of 33 mg of TG-II was also obtained from fraction 4.

ISOLATION OF DIOSCIN [**1**] (TG-I).—The fractions containing TG-I were crystallized from MeOH/Me_2CO to give fine colorless needles, mp 298–300° dec; fabms, $[M+H]^+$ 869 (80%). A sample ot TG-I was compared with an authentic sample of dioscin. There was no melting point depression on admixture (mp 297–300°), their ir spectra were superimposable, their tlc behaviors were identical (R_f = 0.31), and the ^{13}C-nmr data were identical to those previously reported (4).

A small sample (5 mg) of **1** was hydrolysed in 6% HCl in MeOH (2 ml) by heating on a steam bath for 3 h. Upon cooling, the solid was filtered (1 mg) and shown to be diosgenin by tlc, mp, and eims.

ISOLATION OF PENNOGENIN RHAMNOSYL CHACOTRIOSIDE [**3**] (TG-II).—The fractions containing TG-II were crystallized from MeOH/Me_2CO to give fine white crystals, mp 248–250° dec; fabms $[M+H-H_2O]^+$ 1013 (40%); ^{13}C nmr (δ ppm) (aglycone) 37.3 (t), 29.9 (t), 77.9 (d), 38.7 (t), 140.7 (s), 121.5 (d), 32.2 (t), 32.0 (d), 50.0 (d), 36.9 (s), 20.7 (t), 36.9 (t), 44.6 (s), 52.8 (d), 31.8 (t), 89.8 (d), 89.8 (s), 16.8 (q), 19.1 (q), 44.7 (d), 9.0 (q), 109.7 (s), 31.7 (t), 28.5 (t), 30.1 (d), 66.4 (t), 16.9 (q) (C-1 through C-27) glucose moiety 100.0 (d), 79.9 (d), 76.3 (d), 77.3 (d), 78.0 (d), 61.2 (t) (C-1′ through C-6′) rhamnose moiety attached to C-2′ of glucose 101.9 (d), 72.4 (d), 71.9 (d), 73.7 (d), 69.9 (d), 18.4 (q) (C-1″ through C-6″) rhamnose moiety attached to C-4′ of glucose 102.5 (d), 72.4 (d), 72.3 (d), 78.5 (d), 68.2 (d), 18.2 (q) (C-1‴ through C-6‴) rhamnose moiety attached to C-4‴ of rhamnose 101.6 (d), 72.6 (d), 72.0 (d), 73.6 (d), 69.0 (d), 18.0 (q) (C-1⁗ through C-6⁗). A sample of TG-II was compared with an authentic sample of **3**. There was no melting point depression upon admixture (mmp 248–250°), their ir spectra were superimposable, and their tlc behaviors were identical (R_f = 0.39).

EVALUATION OF EFFICACY IN DISSEMINATED CANDIDIASIS.—A mouse model of disseminated candidiasis previously described by Rabinovich *et al.* (12), with some modifications, was utilized for the evaluation of in vivo efficacy. Female ICR mice (Charles River Breeding Laboratories) weighing 20–25 g each were housed in microisolator cages (Lab Products, Maywood, New Jersey) equipped with HEPA filters and were administered food and H_2O ad libitum. Animals were maintained in air-conditioned rooms at 72–74° on a 12 h light, 12 h dark cycle. Animals were quarantined and acclimated 1 week prior to initiation of experiments.

C. albicans strain NIH B311 (13) was used to induce experimental disseminated candidiasis in mice. Cultures of the organism were either lyophilized or stored under sterile mineral oil, and subcultures were prepared on SDA as needed. Subcultures were incubated at 37°. For short-term maintenance, cultures on SDA were stored at 4°. Acute disseminated infections in mice were produced by iv injection (via tail vein) of 10^6 cfu of *C. albicans* NIH B311 in sterile physiological saline solution (PSS). Cell suspensions for injection were prepared by incubation of *C. albicans* B311 in SDB at 37° for 4–6 h, at which time the cells were sonicated briefly (three 5-sec bursts to break chains into single cells for counting and inoculation), centrifuged, washed once, and suspended in PSS to the appropriate dilution, based on direct count by hemacytometer. The reliability of the hemacytometer count was verified by viability determination (by triplicate plating of aliquots of PSS suspension) which showed greater than 96% of the cells as viable cfu. The dose of 10^6 cfu/mouse was verified as lethal within 7–10 days and capable of induction of systemic candidiasis in mice, as evidenced by proliferation of *C. albicans* in the kidneys. Preconditioning of the mice to suppress the immune system was not necessary when log phase (6 h) cultures were used as inoculum.

Seven hours after iv inoculation with *C. albicans*, groups of six mice received varying doses of TG-I and TG-II by ip administration. Doses for TG-I were 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/kg; TG-II was administered at doses of 20, 10, 5, 2.5, 1.0, 0.5 and 0.1 mg/kg. Animals were killed 24 h after infection, and the kidneys were aseptically removed, weighed, and homogenized in 5 ml of PSS. After tenfold serial dilutions from the homogenized kidney suspension (using PSS as diluent), a volume of 0.01 ml was cultured in triplicate on SDA plates which were incubated for approximately 16 h. Cfu were counted to determine the recovery of *C. albicans* from the kidney tissue. The mean numbers of cfu of *C. albicans* per mg of kidney tissue were determined for treated groups and compared to the vehicle-treated infected control group by the Wilcoxon nonparametric rank sum test using $p<0.05$ as a test of significance.

ESTIMATION OF MEDIAN LETHAL DOSE.—The method of Litchfield and Wilcoxon (10) was used to estimate the median lethal dose (LD_{50}) of TG-I and TG-II. Groups of 10 mice (5 male and 5 female) were administered the test compound ip and observed for morbidity and lethality for 14 days. Doses were doubled or halved until the maximal dosage was found, which produced no lethality, and a minimal dosage was found, which produced 100% lethality within a 14-day period. Efforts were made in every case to determine three doses within 0% and 100% mortality range, and an estimated LD_{50} was obtained by probit analysis.

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