VOL. 53 NO. 3, MAR. 2000 THE JOURNAL OF ANTIBIOTICS pp. 256 — 261

# Biological Activity of Guanacastepene, a Novel Diterpenoid Antibiotic Produced by an Unidentified Fungus CR115

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(Received for publication December 24, 1999)

Fermentation extracts of culture CR115, an unknown plant endophyte originally isolated from Costa Rica, were found to be active against antibiotic-resistant bacteria. The metabolite responsible for activity was identified as a novel diterpenoid antibiotic guanacastepene (mol. wt. 374.47 and mol. formula $C_{22}H_{30}O_5$). Mechanistic studies done in an *E. coli imp* strain suggested membrane damage as the primary mode of bactericidal action. This compound also lysed human RBCs and caused leakage of intracellular potassium from *E. coli imp*.

Screening of unusual fungi from various ecological niches around the world has been productive in yielding new bioactive metabolites[1~7]. During our screening of fungal fermentation extracts, an unidentified terrestrial fungus CR115 was found to exhibit antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. This culture was isolated from the branch of a *Daphnopsis americana* in Guanacaste, Costa Rica. On potato dextrose agar (PDA) plate, this culture grew as flat, thin and rippled confluent colony and diffused a light yellow pigment into the agar on maturation. The culture CR115 did not sporulate even after a prolonged incubation period.

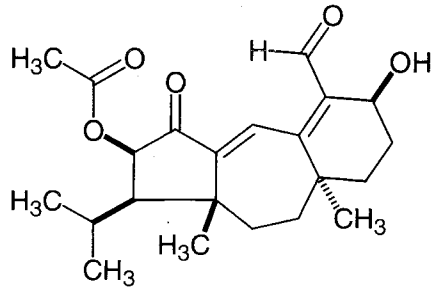
The metabolite responsible for the antimicrobial activity was identified as a novel diterpeneoid antibiotic guanacastepene (Fig. 1)[8]. In the present paper, we report the morphological and growth characteristics of the culture CR115, and antimicrobial activity and the mechanistic studies of this novel diterpene. This work was presented in part at the 38th IAAC held in San Diego[9].

## Materials and Methods

### Culture Maintenance and Growth/Morphological Characteristics

Culture CR115 was maintained on Bennetts' agar plate (incubated at 22°C for 14 days). The culture was streaked on various agar media (see Table 1) and incubated at three different temperatures (22, 28, and 37°C) for up to 21 days. Growth pattern and morphological characteristics were recorded.

Fig. 1. Chemical structure of guanacastepene.




(Molecular formula: $C_{22}H_{30}O_5$, Mass: 374.21, Molecular weoght: 374.47, Elemental analysis: C, 70.56; H, 8.07; O, 21.36)

Fermentation

Two loopfuls of a mature (7~10 days old) plate culture from a Bennett's agar plate was inoculated into a test tube (25×125 mm) containing 10 ml potato dextrose broth (PDB) with 2 glass beads and the tube was incubated for 4 days at 22°C and an agitation rate of 150 rpm. The first seed was then transferred into a 250 ml Erlenmeyer flask containing 50 ml of PDB and further incubated for 4 days at 22°C and 200 rpm. Production medium was inoculated with 5% v/v of the second stage seed.

The first fermentation was carried out in PDB (250 ml per 500 ml Erlenmeyer flask) at 22°C and 170 rpm for 14 days.

Subsequent fermentations were carried out in three liquid (50 ml medium per 250 ml Erlenmeyer flask, incubated at 200 rpm) and three solid media at three different temperatures (22, 28, and 37°C).

*In Vitro* Susceptibility Testing

*In vitro* antibacterial activity was determined by the agar-diffusion method. Assay plates were prepared by pouring 125 ml volume of Mueller-Hinton II agar (tempered at 50°C) inoculated with an overnight broth culture of the test organism to give a density of $10^7$ CFU/ml into a 12″×12″ Sumilon plate. Ten $\mu$l of antibiotic solution (serially diluted in DMSO) was spotted onto the agar surface to give a range of concentrations (200~1 $\mu$g/spot) and the plates were incubated at 37°C for 18 hours. The zone of growth inhibition was measured using a hand-held digital calipers.

The minimum inhibitory concentration (MIC) was determined by the microbroth dilution method[9] in modified minimal medium (contained per liter: dextrose, 4 g; $NH_4Cl$, 1 g; $KH_2PO_4$, 3 g; $Na_2HPO_4$, 6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.5 mg; and vitamin-free casamino acids, 2 g). The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited visible growth of the organism.

Incorporation of Radiolabeled Precursors

Macromolecular synthesis in *E. coli imp* was studied by measuring the incorporation of appropriate radiolabeled precursors into trichloroacetic acid (TCA)-precipitable material[1,10]. *E. coli imp* was grown at 37°C, 200 rpm in modified minimal medium (50 ml medium /250 ml Erlenmeyer flask) to an $A_{450}$ of 0.20. Aliquots of 100 $\mu$l were dispensed into microtiter wells containing antibacterial agents, and the plates were incubated for 10 minutes at 37°C with vigorous agitation. Cells were pulse-labeled for 5 minutes by adding the following radiolabeled precursors at the indicated final concentrations: $^3$H-Tdr, 2 $\mu$Ci/ml with 0.05 $\mu$g unlabeled thymidine/ml; $^3$H-Udr, 2 $\mu$Ci with 2.5 $\mu$g unlabeled uridine/ml; or $^3$H-AA, 2.5 $\mu$Ci/ml. To determine specific incorporation into DNA, RNA, and protein, 100 $\mu$l of chilled (4°C) TCA (10%) supplemented with 0.5 mg of unlabeled precursors per ml was added to each well, and the plate was immediately refrigerated for 1 hour. The precipitate was collected on a glass fiber filter (Wallac filtermat B, Wallac 1205-404) using a Skatron 96-well cell harvester (Model 11050) programmed for a 3 seconds prewet with chilled DI water, a 12 seconds wash with 5% chilled TCA and a 5 seconds drying cycle. Filter mats were dried for 7 minutes at high power in a microwave oven (Quasar, 700 Watts), solid scintillant (MeltilexB, Pharmacia 1205-402) was applied, and the isotope that was retained on the filter was quantitated in an LKB Betaplate scintillation counter (Wallac 1205). The levels of incorporation of $^3$H-Tdr, $^3$H-Udr, and $^3$H-AA were expressed as the percent of the untreated control.

Effect on Intracellular Potassium Level in *E. coli imp*

Effects on the intracellular potassium level in *E. coli imp* was studied in a saline-buffer (10 mM Hepes buffer containing 150 mM NaCl and 0.1 mM KCl, pH 7.0)[1]. A log-phase culture of *E. coli imp* was centrifuged at 3800 rpm at room temperature, supernatant was discarded and the pellet was washed twice with saline-buffer (20 ml) and resuspended in the same buffer to an $OD_{600}$ of 2.00. One milliliter of this bacterial suspension was treated with the test compounds at various concentrations for 1 hour and the cells were pelleted by centrifugation (at 10,000×$g$ at room temperature for 2 minutes). The resulting supernatant was diluted 1 : 10 in HPLC grade water and analyzed for potassium ion by atomic absorption spectrophotometer (Instrumentation Laboratories 551). For the determination of the total potassium level, 1 ml of the culture was hydrolyzed in 2 M sulfuric acid by heat (100°C for 1 hour), chilled for 1 hour, and centrifuged (10,000×$g$ for 2 minutes). The supernatant was collected, diluted 1 : 10 and analyzed for potassium ion.

Lysis of Human RBCs

One milliliter of freshly pooled human blood was centrifuged (at 10,000×$g$ for 2 minutes), the pellet was washed four times with normal saline by repeated resuspension and centrifugation and then the pellet was resuspended in 1 ml of RBC buffer (10 mM Na-phosphate +150 mM NaCl+1 mM $MgCl_2$, pH 7.4)[1]. Twenty five microliter (25 $\mu$l) of the RBC suspension was added to the microfuge tube containing 1 ml of drug solution (final

Table 1. Growth characteristics of culture CR115.

| Medium | Days | Growth at | | | Color at 28°C | | Mycelia at 28°C |
|---|---|---|---|---|---|---|---|
| | | 22° | 28° | 37° | Top | Reverse | |
| CLA | 7 | + | + | none | white | colorless | thin, spreading, some fluff |
| CMA | 7 | + | +++ | +/- | white/beige/orange | yellow-orange | felted to cottony |
| CYA | 7 | + | ++ | +/- | white/beige | light yellow | low, felted |
| GN25 | 7 | none | none | none | | | |
| MEA | 7 | ++ | +++ | + | beige-white | yellow-orange | dense cottony to felted |
| PDA | 7 | + | + | + | white | light yellow | low, fuzzy |
| YePD | 7 | + | ++ | + | white/beige | tan/yellow | felted and fuzzy |
| CLA | 14 | + | + | + | white | colorless | smooth to felted |
| CMA | 14 | + | +++ | + | white/beige/orange | yellow-brown | felted to cottony |
| CYA | 14 | ++ | ++ | ++ | white-beige | cream | low, felted |
| GN25 | 14 | none | none | none | | | |
| MEA | 14 | +++ | +++ | + | beige-white | yellow-orange | dense cottony to felted |
| PDA | 14 | + | ++ | + | white | yellow | rippled, fuzzy |
| YePD | 14 | ++ | +++ | ++ | beige-cream | tan-yellow | felted to floccose |
| CLA | 21 | + | + | + | white | cream | smooth to felted, some cottony |
| CMA | 21 | | +++ | ++ | white/beige/orange | yellow-brown | felted to cottony |
| CYA | 21 | ++ | ++ | ++ | white-beige | cream | low, felted |
| GN25 | 21 | none | none | none | | | |
| MEA | 21 | +++ | +++ | ++ | tan-white | orange-tan | dense cottony to felted |
| PDA | 21 | + | + | + | white | yellow-brown | rippled, fuzzy |
| YePD | 21 | ++ | +++ | +++ | white-yellow | tan-yellow | felted to cottony |

Medium: Carnation leaf agar (CLA), Cornmeal agar (CMA), Czapek-yeast extract agar (CYA), Glycerol nitrate (GN25), Malt extract agar (MEA), Potato dextrose agar (PDA), and Yeast extract peptone dextrose (YePD).

Growth: poor (+), moderate (++), and confluent (+++).

concentration ranging from 1~128 $\mu$g/ml) prepared in duplicate in RBC buffer. After 2 hours of treatment at room temperature, the tube was centrifuged (at 10,000×$g$ for 2 minutes) and the absorbance (at 540 nm) of the supernatant was measured. For 100% lysis as the control, 25 $\mu$l of RBC suspension was added into 1 ml of water.

## Results and Discussion

The fungal culture CR115 was originally isolated from a branch of *Daphnopsis americana* in Guanacaste, Costa Rica. Several growth media were tested for characteristic growth and sporulation; none of the medium supported sporulation even after prolonged incubation period. Bennetts' agar, malt extract agar, corn meal agar and yeast extract peptone dextrose were the media of choice for growth (Table 1). On PDA, the culture grew very low and thin in a rippling pattern and produced a soluble dark yellow pigment on maturation during 7~15 days. The top surface was relatively smooth with some fuzz, mostly white to the edges but the oldest growth was tan. Color on the reverse side of the plate was yellow, but older growth became dark tan in color.

Growth characteristics of the culture CR115 are described in Table 1. The organism grew better at 28°C in all media (Fig. 2 on MEA medium) with the exception of the medium GN25 in which it failed to grow. Culture CR115 produced a soluble yellow pigment on PDA, Bennetts' agar, and YePD media. Colony morphologies

Fig. 2. Growth pattern in the MEA medium after 14 days at (A) 22°C and (B) 28°C.

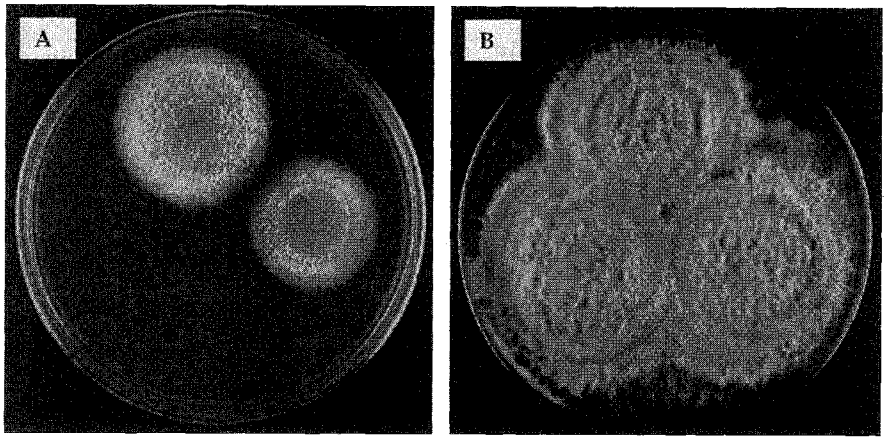



Table 2. Antimicrobial activity of guanacastepene.

| Conc. µg/spot | Sa375 | Sa310 | Ef379 | Ec389 | Ec442 | Ca54 | BIA |
|---|---|---|---|---|---|---|---|
| 50 | 7:10H | 6:9H | 8H | 8H | 5H | 5:9H | 9T |
| 25 | 3:8H | 7H | 6H | 7H | 5H | 8H | 8T |
| 12.5 | 7H | 7H | 6H | 7H | 5H | 7H | 7T |
| 6.25 | 6H | 6H | | 7H | 5H | 6H | 7T |
| 3.1 | 6H | 6H | | 6H | | 6H | 7H |
| 1.6 | 5H | 5H | | 6H | | | 7H |
| 0.8 | | | | 6H | | | 7H |
| 0.4 | | | | 6H | | | 7H |
| **Controls** | | | | | | | |
| Methicillin 5 | 15:22H | 0 | | | | | |
| Vancomycin 30 | 15 | 17 | 0 | | | | |
| Nystatin 100 | | | | | | 16:23H | |
| Bleomycin 5 | | | | | | | 3+ |

Sa: *S. aureus*, Ef: *E. faecium*, Ec: *E. coli*, Ca: *C. albicans*, BIA (Biochemical Induction Assay): detects DNA damaging activity.
Sample solubilized and serially diluted in DMSO at 10 - 0.078 mg/ml. Five microliters (5 µl) of each concentration was spotted on the agar surface.
Plate was incubated for 18h at 37°C and zone of growth inhibition recorded in millimeter.
H, hazy; T, toxic; 3+, strong DNA damage.
MIC (determined by Mircobroth Dilution Method) against *E. coli imp* was 62.5 µg/ml. Guanacastepene was inactive in the BIA.

on different media and different temperatures were considerably different. At 37°C, growth was slow and colonies were compact with darker pigments. Denser or more cottony growth was common at 28°C.

The active metabolite, guanacastepene, was first isolated from PDB (250 ml/500 ml Erlenmeyer flask) fermented for 14 days at 22°C and 170 rpm. Subsequent fermentation studies suggested PDB as the best production medium. The culture grew better at 28°C, but the antibacterial metabolite was produced better at 22°C.

The metabolite responsible for antibacterial activity was isolated from a 14 day fermentation broth (PDB) and was identified as a novel diterpenoid antibiotic guanacastepene (Fig. 1). This compound exhibited moderate activity against a panel of Gram-positive and Gram-negative bacteria and *Candida albicans* (Table 2).

Inhibition of DNA, RNA, and protein synthesis was determined by measuring the incorporation of $^{3}$H-Tdr, $^{3}$H-

Table 3. Effects of guanacastepene and known antimicrobials on the incorporation of radiolabeled precursors into macromolecules in *E. coli imp*.

| Compound | Conc. (μg/ml) | $^3$H-Tdr | $^3$H-Udr | $^3$H-AA |
|---|---|---|---|---|
| Guanacastepene | 128 | 20 | 37 | 27 |
| | 64 | 21 | 44 | 20 |
| | 32 | 93 | 76 | 92 |
| | 16 | 93 | 90 | 100 |
| Ciprofloxacin | 0.25 | 3 | 90 | 95 |
| Rifampin | 0.25 | 93 | 2 | 12 |
| Chloramphenicol | 8 | 98 | 95 | 16 |
| Polymyxin B | 8 | 1 | 2 | 3 |

Data presented represent % of untreated control after 10 min drug pretreatment and 5 min pulse labeling.

Incorporation = precursor incorporated into TCA-precipitable material.

$^3$H-Tdr, $^3$H-Udr and $^3$H-AA are tritiated thymidine, uridine and amino acids, respectively.

Table 4. Membrane damaging effects on human RBC and *E. coli imp*.

| Compound | μg/ml | RBC lysis (%)* | K-Leakage (%)* |
|---|---|---|---|
| Guanacastepene | 128 | 5 | nt |
| | 64 | 29 | 75 |
| | 32 | 83 | 74 |
| | 16 | 92 | 60 |
| | 8 | 90 | nt |
| | 4 | 3 | nt |
| Polymyxin B | 32 | 4 | 60 |
| Amphotericin B | 4 | 60 | nt |
| | 2 | 10 | nt |
| | 1 | 1 | nt |

*Drug treatment periods for RBC lysis and potassium leakage from *E. coli* were 2 and 1h, respectively. nt, not tested.

TUdr, and $^3$H-TAA, respectively, into TCA-precipitable material of a logarithmic-phase culture of *E. coli imp*. Control drugs affected the anticipated macromolecular processes whereas treatment with guanacastepene for 5~10 minutes inhibited incorporation of all three radiolabeled precursors similar to that observed with polymyxin B, suggesting instant disruption of membrane integrity (Table 3). The leakage of intracellular potassium from *E. coli imp* and the lysis of human RBCs further supported the membrane damaging effects of guanacastepene (Table 4). Strongest hemolytic activity was observed at 8~32 μg/ml concentration. Concentrations higher than 64 μg/ml and lower than 8 μg/ml resulted in considerably reduced hemolytic activity.

## Conclusion

Although guanacastepene is a novel diterpenoid antibiotic, its moderate activity against Gram-positive bacteria, poor activity against Gram-negative bacteria and the hemolytic activity against human RBC would limit further development of this compound as an antibacterial agent.

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