The Guanacastepenes: A Highly Diverse Family of Secondary Metabolites Produced by an Endophytic Fungus

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Libraries of structurally diverse "natural product-like" molecules form the basis for understanding biological processes in small molecule-based systematic approaches. Libraries of structurally diverse natural products should be equally useful, and several groups are working on combinatorial biosynthetic approaches to construct such libraries.[1] As part of a recent investigation of an endophytic fungus from Costa Rica, we discovered a remarkable family of structurally diverse diterpenes that provides some insight into how nature constructs libraries of natural products.

Initially we were interested in the natural products of fungus CR115 because at least one of them, guanacastepene A (**1**),[2,3] had antibiotic activity against drug resistant strains of *Staphylococcus aureus* and *Enterococcus faecalis*.[2,4] During the course of that study it became clear that CR115 produces a family of related but structurally diverse metabolites. These metabolites, which are shown in Figure 1, comprise five ring systems (Figure 2) decorated with a variety of functional groups. Additional tautomeric and conformational equilibria in many of the metabolites give still greater structural diversity.

Both a traditional morphological assessment and ribosomal DNA (rDNA) sequencing were performed in an attempt to characterize CR115. CR115 did not produce spores under any conditions tested, and no other morphological characteristics were observed that would suggest its phylogeny.[5] A BLAST search with the sequence derived from the polymerase chain reaction-amplified internal transcribed spacers (ITS1 and ITS2) and the 5.8S rDNA gene did not identify any sequences that were identical to CR115.[6] The most closely related sequence, an uncharacterized oat root Basidiomycete, shows 90% similarity over the ITS1, ITS2, and the 5.8S rDNA regions.[7] This rDNA sequence data, in conjunction with the failure of traditional taxonomic identification, suggests that CR115 likely represents a previously undescribed Basidiomycete.

Guanacastepenes B−O (**2**−**15**) were isolated from the organic extracts of neutralized cultures of CR115 grown in potato dextrose broth. Structures for all of the guanacastepenes were determined by X-ray crystallography[8] and corroborated by high-resolution mass spectrometry.[9] The absolute configurations of guanacastepenes E and L were determined by X-ray crystallography to be as drawn in Figure 1 using anomalous dispersion from the C-5 *p*-bromobenzoyl derivative of each natural product.[10]

The guanacastepenes are more structurally diverse than the individual compounds characterized by X-ray crystallography suggest. Low-temperature NMR and molecular modeling experiments run on guanacastepene A indicate that it exists in two conformations due to conformational flexibility in the C9−C10 bond at the bottom of the central seven-membered ring. This flexibility is further supported by the fact that the C9−C10 bond crystallizes in an alternative conformation in guanacastepene A (C-8−9−10−11 dihedral angle +93.6°) than it does in the other guanacastepenes (C-8−9−10−11 dihedral angles range from −68.4° to −93.0°). NMR experiments also suggest that the hemiacetal in guanacastepene I may exist in both the open and closed forms. As reported in other terpenoids many of the alcohols in the guanacastepene family appear as epimers.[11] Guanacastepenes N and O, C-13 acetate epimers, were isolated and characterized as separate compounds.

Guanacastepene I (**9**) is the only newly characterized guanacastepene with pronounced antibacterial activity in agar diffusion assays against *S. aureus*.[12] On the basis of these limited studies, a C-15 aldehyde, as in guanacastepene A, or a masked C-15 aldehyde, as in guanacastepene I, appears to be a requirement for activity in this assay. Although the other guanacastepenes do not show antibacterial activity at 50 μg per disk, they could be active at higher concentrations, and they are likely to be active in other assays. The spectrum of guanacastepenes produced by CR115 varies from fermentation to fermentation, and this variability has limited the supplies currently available for testing.

The guanacastepenes characterized to date represent only a fraction of the compounds present in the CR115 extract; however, they appear to provide some insight into the origin of this structurally diverse family of natural products. Figure 1 depicts some hypothetical biosynthetic relationships within the guanacastepenes. The tricyclic guanacastepenes A, B, and C are the simplest members of this family of compounds. The successive oxidation and functionalization of these simple compounds or compounds similar to them likely give rise to the more complex ring systems, **17**−**20** (Figure 2). There appear to be two major biosynthetic branches responsible for the formation of more complex guanacastepenes (Figure 1). One group of compounds may arise from an intramolecular Michael addition by the C-15 alcohol at C-2 in guanacastepene C-like precursors (transformation I, Figure 1). A second group of guanacastepenes with more highly oxidized five-membered heterocycles (transformation II) may arise from the intermolecular Michael addition of water (transformation

(1) Khosla, C.; Harbury, P. B. *Nature* **2001**, *409*, 247. Staunton, J.; Wilkinson, B. *Curr. Opin. Chem. Biol.* **2001**, *5*, 159. Cane, D. E.; Khosla, C.; Walsh, C. T. *Science* **1998**, *282*, 63.

(2) Brady, S. F.; Singh, M. P.; Janso, J. E.; Clardy, J. *J. Am. Chem. Soc.* **2000**, *122*, 2116.

(3) Guanacastepene has been given the name guanacastepene A.

(4) Singh, M. P.; Janso, J. E.; Luckman, S. W.; Brady, S. F.; Clardy, J.; Greenstein, M.; Maiese, W. M. *J. Antibiot.* **2000**, *53*, 256.

(5) Analytical Services, Inc. (Williston, VT) performed the taxonomic studies on CR115.

(6) rDNA was amplified from crude CR115 DNA with primers ITS1 and ITS4 using the polymerase chain reaction and then the gel-purified PCR product was directly sequenced with ITS1, ITS2, ITS3, and ITS4. (White, T. J.; Bruns, T.; Lee, S. Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols. A Guide to Methods and Applications*; Innins, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., Eds.; Academic Press: San Diego, CA, 1990; pp 315−322.) The sequence used in the BLAST search was derived from sequencing four independent PCR products.

(7) Carter, J. P.; Spink, J.; Cannon, P. F.; Daniels, M. J.; Osburn, A. E. *Appl. Environ. Microbiol.* **1999**, *65*, 3364.

(8) Crystallographic data for **2**−**15** and the C-5 *p*-bromobenzoyl derivatives of **5** and **12** have been deposited with the Cambridge Crystallographic Data Center.

(9) HRMS-FAB (*m/z*) $[M + H]^+$: guanacastepene B (**2**) calcd for $C_{20}H_{33}O_3$ 321.2430, found 321.2421; C (**3**) calcd for $C_{20}H_{31}O_4$ 335.2222, found 335.2221, D (**4**) calcd for $C_{22}H_{28}NO_3$ 354.2069, found 354.2068; E (**5**) calcd for $C_{22}H_{33}O_5$ 377.2328, found 377.2328; F (**6**) calcd for $C_{22}H_{31}O_6$ 391.2121, found 391.2121; G (**7**) calcd for $C_{22}H_{31}O_5$ 375.2171, found 375.2171; H (**8**) calcd for $C_{22}H_{30}NO_5$ 388.2124, found 388.2125; I (**9**) calcd for $C_{21}H_{31}O_5$ 363.2171, found 363.2170; J (**10**) calcd for $C_{21}H_{29}O_5$ 361.2015, found 361.2016; K (**11**) calcd for $C_{22}H_{29}O_6$ 389.1964, found 389.1964; L (**12**) calcd for $C_{20}H_{25}O_5$ 345.1702, found 345.1702; M (**13**) calcd for $C_{20}H_{23}O_5$ 343.1545, found 343.1547; N (**14**) calcd for $C_{22}H_{29}O_6$ 389.1964, found 389.1964; O (**15**) calcd for $C_{22}H_{29}O_6$ 389.1964, found 389.1964.

(10) The absolute structure factors for the C-5 *p*-bromobenzoyl derivatives of **5** and **12** are 0.023 (6) and 0.16 (5), respectively, for the absolute configuration of guanacastepenes E and L shown in Figure 1.

(11) Wise, M. L.; Croteau, R. Monoterpene Biosynthesis. In *Comprehensive Natural Products Chemistry*; Barton D., Nakanishi, K., Meth-Cohn, O., Cane, D. E., Eds.; Elsevier: Amsterdam, 1999; Vol. 2, pp 143−147.

(12) Guanacastepene J (**10**) could not be accurately assayed because of the limited quantity available.

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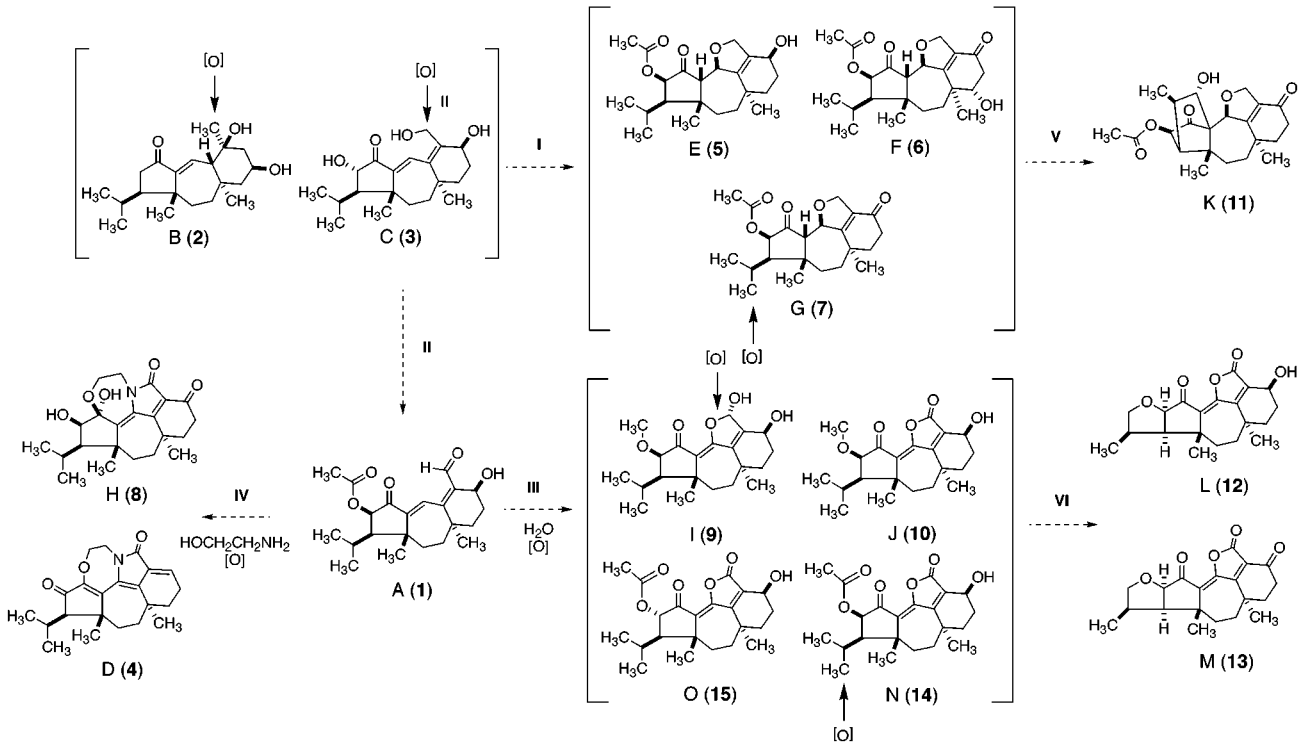

Figure 1. Guanacastepenes A−O are organized to show possible biosynthetic relationships within this family of natural products. A few oxidations and transformations (I−VI) that could explain the origin of each ring system have been highlighted. The absolute configuration of guanacastepenes E and L was determined to be as it is shown.

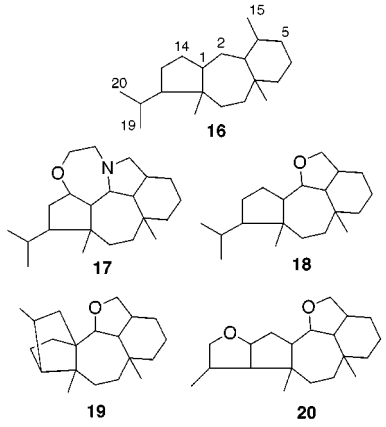

Figure 2. Five ring systems produced and functionalized by CR115 to create the guanacastepenes.

III) or ethanolamine (transformation IV) at C-2 in guanacastepene A-like precursors followed by the reoxidation of the C-1/C-2 double bond. Both families then appear to undergo similar isopropyl methyl oxidations to give either the norbornane-containing ring system **19** (transformation V) seen in guanacastepene K (**11**) or the pentacyclic ring system **20** (transformation VI) seen in guanacastepenes L and M. The novel diterpene carbon skeleton **19** likely results from the oxidation of an isopropyl methyl (C-19/C-20) to an aldehyde followed by an aldol condensation at C-1. In addition to the important ring generating biosynthetic transformations highlighted in Figure 1, there also appear to be a number of traditional terpenoid oxidation/reduction and conjugation (methylation and acetylation) pathways involved in the biosynthesis of the guanacastepenes.

The chemical diversity of the guanacastepenes could either arise from multiple biosynthetic pathways working in concert or a single highly branched biosynthetic grid analagous to synthetic libraries of natural product-like compounds that are derived either from parallel syntheses or from large split and pool syntheses. It is likely that the structural diversity of the guanacastepenes represents CR115's attempt to maximize the molecular diversity of its chemical arsenal while minimizing metabolic costs.[13] Understanding the details of how CR115 solves this optimization problem and comparing this solution with laboratory solutions should provide interesting insights. Genetic and biochemical studies currently underway should help elucidate the biosynthetic mechanism(s) that lead to the creation of this structurally diverse family of natural products.

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Supporting Information Available: Computer generated perspective drawings from the X-ray crystallography experiments, ribosomal DNA sequence derived from CR115, archival X-ray tables, ^{13}C NMR spectra and ^{1}H NMR spectra. (PDF) This material is available free of charge via the Internet at http://pubs.acs.org.

JA016176Y

(13) Jones, C. G.; Firn, R. D. *Philos. Trans. R. Soc. London, Ser. B* **1991**, *333*, 273.