

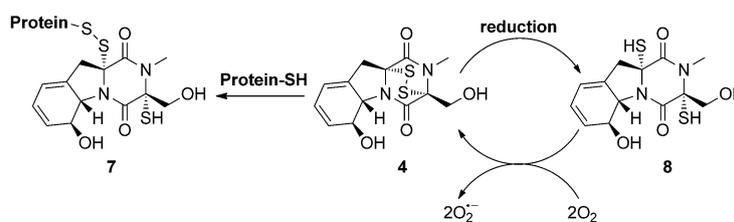
Gliotoxin: Nature's Way of Making the Epidithio Bridge**

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biosynthesis · bridged alkaloids · disulfide · epipolythiodioxopiperazine · gliotoxin

Alkaloids containing a central bicyclo[*n*.2.2]piperazinedione (diketopiperazine, DKP, $n \geq 2$) ring system, bridged at positions 3 and 6, constitute an ever-growing class of secondary metabolites. DKPs **1**,^[1] biosynthesized from amino acids either by nonribosomal peptide synthases (NRPS) or by tRNA-dependent enzymes called cyclodipeptide synthases (CDPS),^[2] are the central precursors, from which diverse alkaloids with different bridge types and sizes arise through further enzymatic transformations (Figure 1). Such alkaloids include the prenylated indole alkaloids such as brevianamide A (**2**)^[3] and the unusual antibiotic bicyclomycin (**3**).^[4] Oxidative modifications of **1** also provide structurally diverse natural sulfur compounds named epipolythiodioxopiperazines (ETPs), such as gliotoxin (**4**) or other higher order ETP alkaloids, such as **5** or **6**.^[5]

Gliotoxin (**4**), isolated in 1943, was the first member of the ETP class and bridged DKP alkaloids in general.^[6] Its structure remained incorrectly assigned until 1958, when Woodward and co-workers suggested the correct, but unprecedented at that time, structure incorporating a disulfide bridge.^[7] The wide range of biological properties, such as antiviral, antibacterial, and immunosuppressive activities, are all presumed to be a direct consequence of the reactivity of the epidithio bridge (Scheme 1), which is capable of generat-



Scheme 1. The bioactivity of gliotoxin associated with its epidithio bridge.

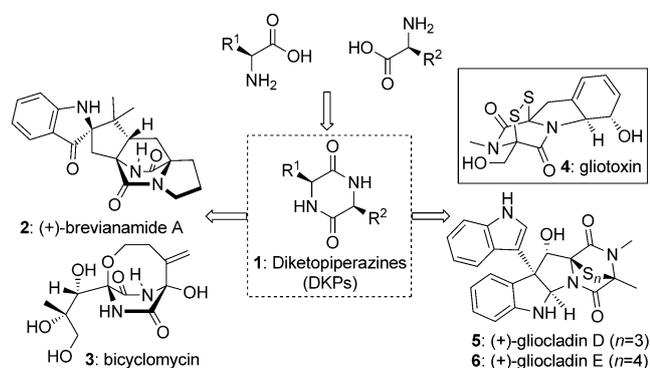


Figure 1. Diversity of bridged diketopiperazine alkaloids.

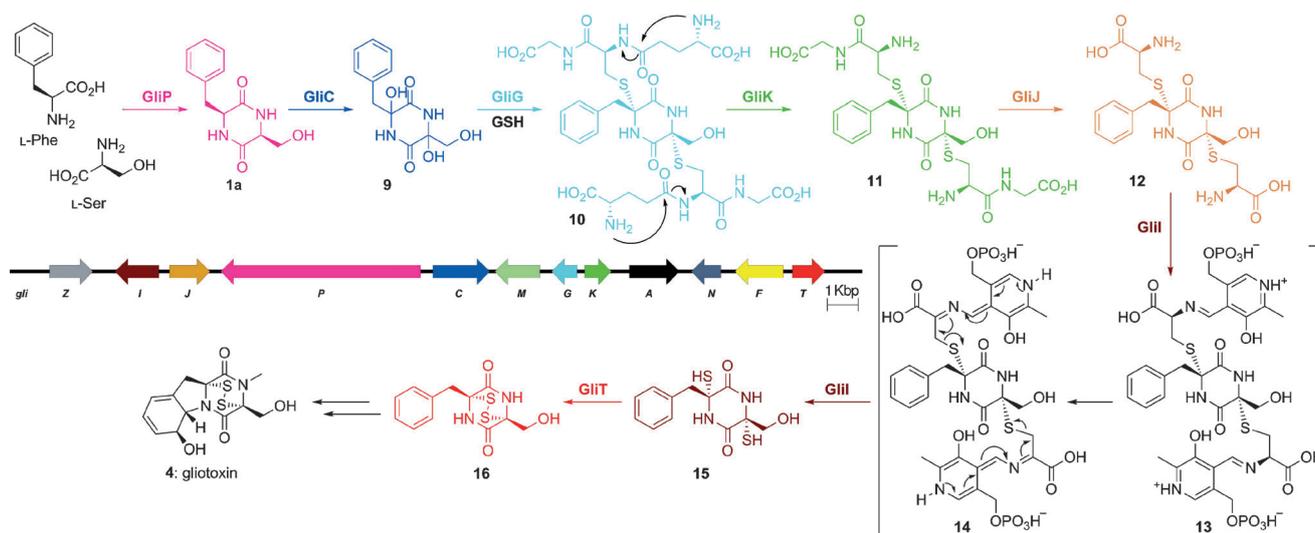
ing reactive oxygen species and engaging in protein conjugation.^[8]

Despite the increasing importance of the ETP alkaloid class, little progress was made in understanding the biosynthesis of **4** and related natural products. Limitations of the classical labeling and feeding approaches allowed no further insight than the involvement of the amino acids phenylalanine and serine as the precursors to the key DKP intermediate cyclo(L-Phe-L-Ser) (**1a**, $R^1 = \text{benzyl}$, $R^2 = \text{hydroxymethyl}$).^[9] The most interesting questions in the biosynthesis of gliotoxin, concerning the mode of incorporation of the sulfur atoms and the way the epidithio bridge is formed, could however not be unambiguously answered and remained at the level of hypotheses for over half a century. The ease of formation of the epidithio bridge by air oxidation of DKP-derived *cis*-dithiols, as confirmed by many synthetic studies, poses the intriguing question of whether it is a spontaneous or an enzyme-catalyzed transformation.

Over the last two decades, a paradigm shift in the elucidation of the biosynthesis of natural products toward connecting the secondary metabolites to their encoding genes revolutionized the field.^[10] Instead of synthesizing isotopically labeled precursors and intermediates, the genes and enzymes encoded by them became the targets. The molecular genetics

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Scheme 2. The confirmed key steps in the biosynthesis of gliotoxin. Inset: gliotoxin gene cluster of *Aspergillus fumigatus*.

approach provides much deeper insight into nature's chemical craftsmanship and has already led to flourishing new research areas such as combinatorial biosynthesis,^[11] chemoenzymatic total synthesis, and mutasynthesis.^[12]

The full genome sequencing of *Aspergillus fumigatus* in 2005^[13] enabled the identification of the genes that are responsible for the biosynthesis of gliotoxin by genome mining.^[14] Thus, the gliotoxin gene cluster (Scheme 2, inset), which consists of 12 genes, became the target of study by several research groups in a race to elucidate its biosynthesis. Balibar and Walsh showed in 2006 that the largest of these genes, *gliP*, codes for a multimodular NRPS. The derived GliP enzyme catalyzes the formation of the DKP cyclo(L-Phe-L-Ser) (**1a**), thus confirming the half century old results of feeding experiments.^[15]

Now, in a series of seminal publications, Hertweck and co-workers achieved the elucidation of all the key steps in the biosynthesis of gliotoxin, from the introduction of the sulfur atoms into **1a** to the formation of the epidithio bridge in **16**, by using genomic information and molecular biology techniques.^[16]

In 2010 the Hertweck^[16a] and Doyle^[17] research groups independently showed that the formation of the disulfide bond in gliotoxin is catalyzed by an FAD-dependent homodimeric enzyme GliT, which uses molecular oxygen as the stoichiometric oxidant to convert the dithiol form **8** into gliotoxin **4** (cf. Scheme 1).

The Hertweck research group demonstrated by both in vitro and in vivo assays that a twofold hydroxylation of DKP **1a** by GliC, a cytochrome P450 monooxygenase, is a prerequisite for subsequent sulfuration of **9** by GliG, a dedicated glutathione S-transferase,^[16b] whose role was also independently established by Doyle and co-workers.^[18] Although no information about the configuration of the dihydroxy DKP **9** was reported, the incorporation of glutathione proceeds with *cis* selectivity. The resulting *cis*-bis(glutathione) DKP **10** was isolated and fully characterized by creating mutant strains lacking the gene *gliK*, which codes for

a γ -glutamate cyclotransferase GliK that is responsible for processing **10** to an intermediate Cys-Gly adduct **11** and pyroglutamate.^[16d] Subsequently, GliJ was identified as a dipeptidase, which removes the two glycine fragments to generate the unstable and short-lived bis(cysteinylyl) DKP **12**. When the *gliI* gene was deleted, mutants incapable of producing gliotoxin resulted. However, this effort led to the isolation of 1.49 mg of **12** from 140 L of mutant culture mycelia and allowed its full characterization. Significantly, the incubation of **12** with the purified GliI enzyme, which was found to be a homodimeric pyridoxal phosphate dependent C-S lyase, led to conversion of **12** into epidithiol **15**, presumably via **13** and **14**, along with its air-oxidized epidisulfide **16**.^[16c]

Most spectacularly, after isolation and characterization of the complete enzymatic machinery for the transformation of DKP **1a** to the epidisulfide **16**, Hertweck and co-workers demonstrated that **16** was also formed by incubating the bis(glutathione) adduct **10** in vitro with a mixture of the enzymes GliK, GliJ, GliI, and GliT in a one-pot four-enzyme cascade. Furthermore, GliT was found to be capable of forming the disulfide bridge from **15** as well as from **8**.^[16d]

Very recently the X-ray structures of GliT as well as two related thiol oxidases were solved by Huber and Groll in collaboration with the Hertweck group. This allowed the formulation of a unified mechanism for the formation of the disulfide bridge.^[19]

These studies allowed the step-by-step reconstitution of the entire sequence of key transformations associated with the biosynthesis of the disulfide bridge of gliotoxin. A deep insight into the incorporation of the sulfur atoms into secondary metabolites was obtained with the characterization of GliG as the first characterized dedicated glutathione transferase. The results have biological consequences. For example, the resistance of the pathogenic microorganism *Aspergillus fumigatus* to its own toxin may follow directly from the dual action of the key enzyme GliT catalyzing the formation of the disulfide bridge in **4** from **8** as well as in **16**

from **15**,^[16a,d,17] thus avoiding the generation of reactive oxygen species and nonspecific protein conjugates. These results will have medicinal implications. After the solving of the X-ray structure, GliT can now be identified as the Achilles heel of the pathogen and the design of drugs for treating invasive aspergillosis will be facilitated, since gliotoxin is believed to be the main virulence factor of *Aspergillus fumigatus*. Overall, the story of gliotoxin biosynthesis demonstrates how natural product research has radically changed with the help of bioinformatics, molecular biology, biotechnology, and modern analytical methods.

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