Biosynthesis and Synthesis of 3-Benzyl-6-ethyl-3,6-bis(methylthio)-piperazine-2,5-dione, an 'Unnatural' Metabolite of *Gliocladium deliquescens*

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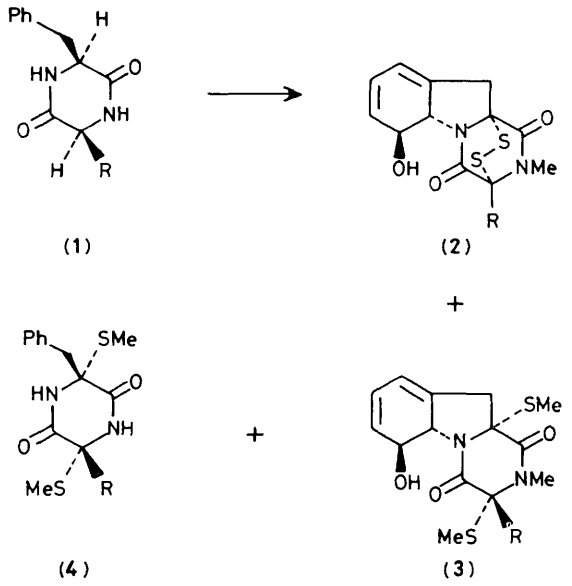
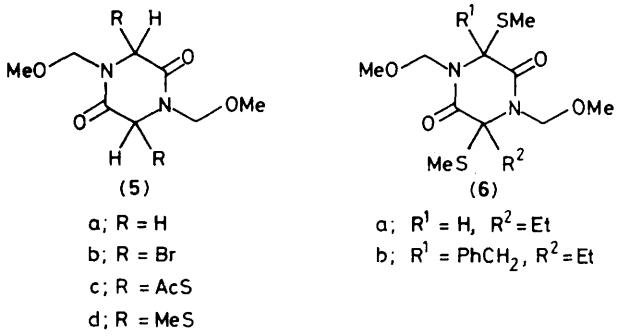
cyclo-(L-2-Aminobutanoyl-L-phenylalanyl) (**1**; R = Et), an unnatural analogue of the gliotoxin (**2**; R = CH_2OH) precursor, *cyclo*-(L-Phe-L-Ser) (**1**; R = CH_2OH), is transformed in *Gliocladium deliquescens* into the titled compound (**4**; R = Et) showing that biosynthetic introduction of sulphur may proceed without prior *N*-methylation or oxidative cyclisation of the phenyl group.

Cyclodipeptides [as (**1**)] derived from L-amino-acids have been shown to serve as biosynthetic precursors for sulphur-containing metabolites from several fungi.[1] However, nothing is known of the order of biosynthetic steps leading from these simple precursors to the complex end products. For example, *cyclo*-(L-Phe-L-Ser) (**1**; R = CH_2OH) is converted,[2] in *Gliocladium deliquescens*, into gliotoxin (**2**; R = CH_2OH), but no intermediates have been detected and the sequence of the major operations, *viz.* *N*-methylation, oxidative cyclisation on to the phenyl ring, and introduction of sulphur, remains undefined. Also, *cyclo*-(L-Ala-L-Phe) (**1**; R = Me) is transformed[3] biosynthetically into the 'unnatural' metabolite 3a-deoxygliotoxin (**2**; R = Me) with an efficiency comparable with that of the 'natural' process but, again, no intermediates were detected. We now report feeding experiments with *cyclo*-(L-2-aminobutanoyl-L-phenylalanyl) (**1**; R = Et) which have led to the identification of a new, 'primitive' type (**4**; R = Et) of sulphur-containing metabolite.

The cyclodipeptide (**1**; R = Et), prepared in radiolabelled form from *N*-benzyloxycarbonyl-L-2-aminobutyric acid and





L-[U-^{14}C]phenylalanine methyl ester in the usual way, was fed in dimethyl sulphoxide to cultures of *Gliocladium deliquescens* (NRRL 1828) under the standard conditions.[2] The chloroform extract of the culture filtrates was freed from most of the gliotoxin by crystallisation and the mother liquors were chromatographed on silica plates developed with toluene–acetone (2:1). Radio-scanning and autoradiography revealed 3 radioactive bands of higher R_f than gliotoxin, designated for discussion as fractions I, II, and III in descending R_f value. These fractions contained similar amounts of radioactive material (each *ca.* 4% of the radioactivity fed) and the radioactive components were readily separated from each other. However, all the fractions contained (n.m.r. control) other components, presumably not derived from (**1**; R = Et), and repeated chromatography failed to yield pure samples of the new metabolites. Nevertheless, ^{1}H n.m.r. spectroscopy permitted tentative structures to be assigned as (**2**; R = Et) (in fraction I), (**3**; R = Et)† (in fraction II), and (**4**; R = Et) (in fraction III). Assignments were greatly assisted by the observation of triplets (*J* 7 Hz) at δ ($CDCl_3$) 1.28, 0.74, and 0.31, for fractions I, II, and III respectively, which could only have arisen from ethyl groups in metabolites of the precursor (**1**; R = Et). Since compound (**4**; R = Et) was of a new, and biosynthetically significant, type a firm structural proof was sought by the following synthesis. The methods were based closely on those developed by Kishi *et al.*[5]

The derivative (**5a**) was prepared either from glycine anhydride by successive treatment with formaldehyde and methanolic hydrogen chloride,[6] or from *N*-acetylglycine anhydride with methoxymethyl chloride and potassium t-butoxide. The derivative (**5a**) was converted, in the usual way,[5] *via* the dibromo-compound (**5b**), into the bis(acetylthio)-compound (**5c**), which was obtained, after crystallisation, as a single stereoisomer‡ [50% from (**5a**)], m.p. 132—135 °C. Treatment of (**5c**) with potassium hydroxide in ethanol containing iodomethane then gave (50%) the bis(methylthio)-derivative (**5d**), m.p. 91—92 °C. *C*-Ethylation of (**5d**) was effected with lithium di-isopropylamide (LDA) (2.5 mol. equiv.) and iodoethane (10 mol. equiv.) in tetrahydrofuran (THF) at −78 °C for 20 h to give (**6a**) (50%), m.p. 63—64 °C. This product was benzylated using LDA (1.25 mol. equiv.) and benzyl bromide (5 mol. equiv.) in THF, initially at −78 °C and then at −11 °C for 20 h, to give (75%) (**6b**) as an oily mixture of *cis*- (85%), δ($CDCl_3$) 0.08 (t, *J* 7 Hz, CH_2CH_3), and *trans*-isomers (15%), δ($CDCl_3$) 0.93 (t, *J* 7 Hz, CH_2CH_3). Deprotection of the mixture (**6b**) was effected with boron tribromide (2.2 mol. equiv.) in dichloromethane at −78 °C followed by hydrolysis of the resulting complex mixture with sodium hydrogen carbonate in aqueous acetone at room temperature to give the *cis*-isomer [racemate of (**4**)] (40%), m.p. 287 °C; δ($CDCl_3$, 100 MHz) 0.31 (t, *J* 7.4 Hz, CH_2CH_3), 1.33—1.75 (*m*, CH_2CH_3), 2.23 (s, SMe), 2.39 (s, SMe), 2.95 and 3.65 (ABq, *J* 13.6 Hz, $PhCH_2$), 5.71 (br. s, NH), 5.99 (br. s, NH), and 7.25 (m, phenyl-H). The n.m.r. spectrum of the racemate was identical, in all relevant details, with that of the impure metabolite (**4**). Final proof of the structure (with the exception of absolute configuration) of the metabolite was obtained by radio-dilution. A sample of fraction III, containing radio-labelled (**4**), was diluted with an excess of the synthetic racemate. Repeated recrystallisation gave racemic (**4**) with a constant specific activity corresponding to 63%§ of the activity originally in the impure metabolite.

Clearly, replacement of the hydroxymethyl group of (**1**; R = CH_2OH) by an ethyl group causes a significant disturbance in the relative rates, and associated pool sizes, of the steps in the biosynthetic pathway leading to sulphur-containing metabolites in *G. deliquescens*. This effect was sought but not observed[3] with the simpler, unnatural precursor (**1**; R = Me). The formation of (**4**), the first known example of this 'primitive' type shows that the organism is able to introduce sulphur before *N*-methylation or oxidative modification of the phenyl group. Provisionally, we suggest that (**4**) arises by *S*-methylation of the corresponding bisthiol, which accumulates to unnatural levels by retardation of subsequent steps in the normal biosynthetic pathway. Supporting evidence, from experiments with a different fungus, for early introduction of sulphur is presented in the following paper.[7]

† The corresponding hydroxymethyl compound (**3**; R = CH_2OH) is a known[4] metabolite of *G. deliquescens* but is only present in small amounts (*ca.* 6% of the amount of gliotoxin).

‡ The stereochemistry of compounds (**5b**—**d**) and (**6a**) was not determined; for a discussion of stereochemistry in related series see ref. 5.

§ Radiochemical purity was achieved rapidly by repeated crystallisation. The loss of 37% total activity may be attributable to the presence of another, unidentified metabolite or, more probably, to decomposition products resulting from long storage of fraction III prior to dilution analysis.

We thank the S.E.R.C. for financial support (to M. A. S. and R. R. T.), the Commonwealth Scholarship Commission for a Fellowship (to P. S. R.), and C. H. Boehringer Sohn (Ingelheim) for leave of absence (to W. L.).

Received, 3rd May 1983; Com. 546

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