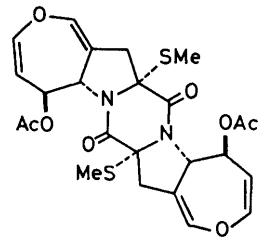
Biosynthetic Incorporation of *cyclo*-(L-Phenylalanyl-L-phenylalanyl) into Bisdethiobis(methylthio)acetylaranotin in *Aspergillus terreus*

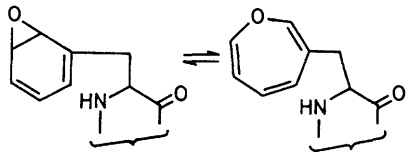
By MARIA I. PITA BOENTE, GORDON W. KIRBY,* and DAVID J. ROBINS
(*Department of Chemistry, University of Glasgow, Glasgow* G12 8QQ)

Summary *cyclo*-(L-[^{15}N]Phenylalanyl-L-[1-^{13}C]phenylalanyl) was incorporated intact into bisdethiobis(methylthio)acetylaranotin (**1**) in *Aspergillus terreus*, the presence of doubly labelled species in the product being detected by ^{13}C n.m.r. spectroscopy (J_{CN} 13 Hz).

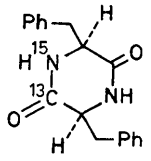
cyclo-(L-PHENYLALANYL-L-SERYL) is an efficient biosynthetic precursor of gliotoxin in *Gliocladium deliquescens*, incorporation occurring without detectable separation and recombination of the amino-acid units.[1] More recently, the incorporation of *cyclo*-(L-seryl-L-[*U*-^{14}C]tyrosyl) and the corresponding aryl dimethylallyl ether into sirodesmin PL in *Phoma lingam* has been described.[2] We report here our studies on the biosynthesis of the aranotin group of metabolites using various stereoisomers and labelled forms of *cyclo*-(phenylalanylphenylalanyl).



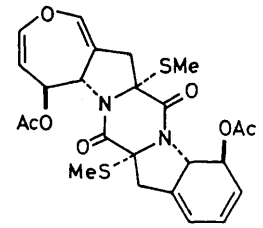
(1) BDA



(2)



(3)



(4) BDAA

Bisdethiobis(methylthio)acetylaranotin (BDA) (**1**),[3] a major metabolite of *Aspergillus terreus* (NRRL 3319),[4] was selected for detailed study. The oxepin ring systems in (**1**) are believed to arise *via* ring-opening of arene oxides [as (**2**)] followed by epoxidation and cyclisation.[5] Incorporation of phenylalanine into BDA (**1**) has been observed[5] but no later intermediate on the biosynthetic pathway has previously been identified. *cyclo*-(L-[*U*-^{14}C]Phe-L-Phe) [as (**3**) but without ^{15}N and ^{13}C enrichment] (33 mg), prepared by standard methods,[6] in dimethyl sulphoxide (19 ml), was added to cultures of *A. terreus* (2·4 l) 48 h after inoculation. The cultures were harvested 5 days later and the major metabolites, BDA (**1**) and bisdethiobis(methylthio)acetylapoaranotin (BDAA) (**4**), were isolated by extraction with chloroform and purified by layer chromatography. Radioscanning and autoradiography of t.l.c. plates indicated incorporation of ^{14}C into both BDA and BDAA but only the former was obtained rigorously pure and crystallised to constant specific activity. Incorporation of *cyclo*-(L-Phe-L-Phe) into BDA (**1**) was found to occur with high efficiency (19·9% incorporation) and low dilution (4·6). In contrast, neither *cyclo*-(L-Phe-D-Phe) nor *cyclo*-(D-Phe-D-Phe) served as an efficient precursor of BDA (**1**). Thus, when a mixture of *cyclo*-(L-[1-^{14}C]Phe-D-Phe) (60%) and *cyclo*-(D-[1-^{14}C]Phe-D-Phe) (40%) was fed to *A. terreus* the resulting BDA (**1**) contained little ^{14}C (incorporation 0·5%, dilution 94). It appeared therefore that only the LL-*cyclo*-dipeptide merited further consideration as an intermediate on the biosynthetic pathway. Confirmation of the status of *cyclo*-(L-Phe-L-Phe) as a natural intermediate was obtained from a trapping experiment. Non-radioactive *cyclo*-(L-Phe-L-Phe) was incubated with *A. terreus* and, 2 h later, L-[*U*-^{14}C]phenylalanine was added to the culture medium. Incubation was stopped after a further 2 h and the culture medium was extracted with chloroform. Concentration of the extract caused separation of *cyclo*-(L-Phe-L-Phe). This was recrystallised to a constant specific activity corresponding to a 10·4% incorporation of the activity administered as L-[*U*-^{14}C]phenylalanine.

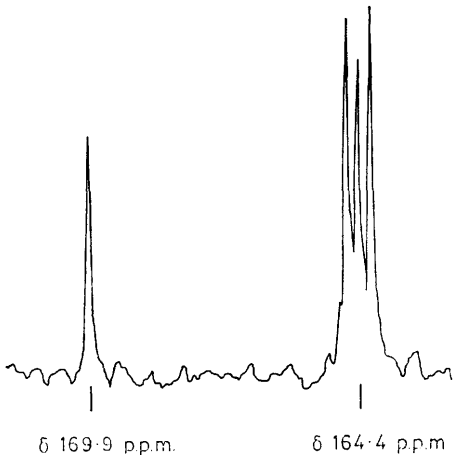


FIGURE. The {^{1}H} ^{13}C n.m.r. spectrum ($CDCl_3$, 100 MHz) of BDA (**1**) derived from *cyclo*-(L-[^{15}N]Phe-L-[1-^{13}C]Phe) (**3**). At natural ^{13}C abundance, the height of the amide carbonyl signal (δ 164·4 p.p.m.) was 73% that of the acetate carbonyl signal (δ 169·9 p.p.m.).

The foregoing results do not exclude the possibility that *cyclo*-(L-Phe-L-Phe) is interconverted, in *A. terreus*, with phenylalanine or a biochemically equivalent, 'monomeric' species and might, therefore, not lie on the main biosynthetic pathway to BDA (**1**). To establish intact incorporation of the symmetrical *cyclo*-dipeptide we selected the species (**3**), doubly labelled at high atomic abundance with ^{13}C and ^{15}N, in the expectation that ^{13}C–^{15}N coupling[7] would be observable in the ^{13}C n.m.r. spectrum of the derived BDA. A mixture of *cyclo*-(L-[^{15}N]Phe-L-[1-^{13}C]Phe) (**3**) and *cyclo*-(L-[^{15}N]Phe-D-[1-^{13}C]Phe) was prepared from L-[^{15}N]-phenylalanine (96·5% ^{15}N) and DL-[1-^{13}C]phenylalanine (91% ^{13}C). To this was added *cyclo*-(L-[*U*-^{14}C]Phe-L-Phe), to provide an independent measure of dilution, and unlabelled *cyclo*-(L-Phe-L-Phe), to ensure that adequate mixing of labelled and unlabelled 'monomeric' units would occur if the *cyclo*-dipeptide were to dissociate reversibly in the organism. This precursor mixture was fed to *A. terreus* under the usual growth conditions and the resulting BDA was crystallised to constant activity (incorporation 14·4%, dilution 6·0) and examined by ^{13}C n.m.r. spectroscopy. The Figure shows the acetate (δ 169·9 p.p.m.) and dioxopiperazine (δ 164·4 p.p.m.) carbonyl signals for the labelled BDA; the remainder of the spectrum was indistinguishable from that of BDA (**1**) containing ^{13}C at natural abundance. Clearly, the BDA was substantially enriched with ^{13}C in the amide carbonyl groups and at no other site. Also, the presence of directly labelled ^{13}C and ^{15}N nuclei in the BDA was evident from the doublet splitting (*J* 13 Hz) of the amide

carbonyl signal. The central component of the amide triplet can arise only from ^{13}C–^{14}N labelled molecules. Its height† was used, as follows, to estimate the maximum extent to which dissociation of the precursor might have occurred during biosynthesis. The height expected for intact incorporation of the precursor was calculated from the measured (^{14}C) dilution and known ^{13}C–^{14}N composition of the precursor. The calculated height was 83% of that measured from the spectrum, after correction for overlap of the flanking, doublet signals. This discrepancy might merely reflect accumulated experimental errors but could have resulted from partial dissociation of the precursor. The latter possibility, which was central to the purpose of the experiment, was examined quantitatively. Calculation showed that a 5% dissociation of the precursor molecules and random recombination of the resulting fragments‡ would account in full for the observed signal height.

We conclude that essentially intact incorporation of *cyclo*-(L-Phe-L-Phe) into BDA (**1**) occurs in *A. terreus*.

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† The intensity of the doublet signals depends on the relaxation mechanism for ^{13}C nuclei directly bonded to ^{15}N. A direct comparison with natural abundance ^{13}C signals (^{13}C–^{14}N) is not valid.

‡ In the calculation it was assumed that the fragments would not be diluted by equivalent, unlabelled, endogenous units. In consequence, the 5% dissociation represents the maximum needed to accommodate the experimental result.

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