

## Biosynthesis of the Thiazolylpeptide Antibiotic GE2270

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The biosynthesis of the antibiotic GE2270 in the producing microorganism *Planobispora rosea* was investigated by adding labelled amino acid precursors. Efficient incorporation of glycine and serine was observed, leading to specific enrichments of selected positions of the thiazole, oxazoline and pyridine rings. Furthermore, efficient enrichment of the C-, N- and O-methyl groups was detected. These results indicate that GE2270 is made through a biosynthetic route similar to that determined for other thiazolylpeptides. At the same time, the results point to an efficient route for the conversion of glycine into serine and methyl equivalents in *Planobispora rosea*.

GE2270, a potent antibiotic active against Gram-positive bacteria<sup>1)</sup>, is characterized by a highly modified peptide backbone consisting of six thiazole, one pyridine and one oxazoline rings (Fig. 1). This compound is thus structurally related to other thiazolylpeptide antibiotics (thiostrepton, nosiheptide, etc.) that also consists of a highly modified peptide with several thiazole rings. While all thiazolylpeptides exert their action by inhibiting bacterial protein synthesis, GE2270, together with the closely related molecules amithiamycin<sup>2)</sup> and GE37468<sup>3)</sup>, is a potent and selective inhibitor of bacterial elongation factor Tu<sup>4-6)</sup>, and thus differs in its molecular target from thiostrepton and related compounds, which bind to the L11 protein in the 23S ribosomal complex<sup>7)</sup>. The biosynthesis of thiazolylpeptide antibiotics has been investigated in several instances<sup>8-12)</sup>. These studies have shown that the thiazole rings are labelled by serine, likely after its conversion to cysteine, and that the pyridine ring, also present in the thiostrepton family of antibiotics, derives from the head to head condensation of two serine residues<sup>13)</sup>. By analogy to these molecules, GE2270 is expected to be derived from six cysteine residues (to yield thiazole rings A through F), three serine residues (two for the pyridine ring and one for the oxazoline), and one residue each of asparagine, valine, glycine, phenylalanine and proline.

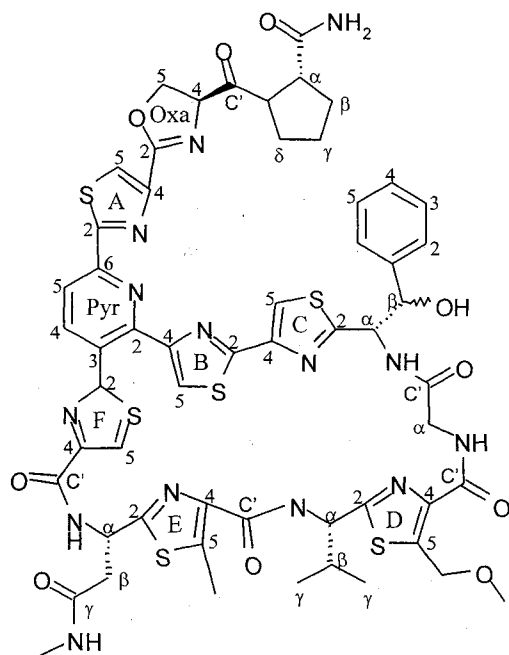
GE2270 is produced by *Planobispora rosea*, one of the lesser studied genera of actinomycetes<sup>14)</sup>. In fact, no other secondary metabolites have been described from this genus. Furthermore, this antibiotic is produced as a complex of several related compounds, differing mostly in the degree of methylation<sup>15,16)</sup>. Two aspects warranted an investigation of GE2270 biosynthesis through the use of labelled precursors: the origin of the C-methyls in thiazoles C and D (Fig. 1), and a first characterization of antibiotic biosynthesis in a *Planobispora* sp. These studies are reported herein.

### Material and Methods

The *Planobispora rosea* strain used in this work was strain GE43782 (from Biosearch Italia strain collection), a GE2270-high-producing derivative obtained by mutagenesis of the parental strain ATCC 53733. The vegetative medium was D-Seed/2 (0.5% peptone, 0.3% yeast extract, 0.2% beef extract, 0.2% soybean meal, 2% starch, 1% calcium carbonate, pH 7.0), while the production medium was MC-FP (2% starch, 0.25% peptone, 0.25% hydrolyzed caseine, 0.3% yeast extract, 0.2% beef extract, 0.2% soybean meal, 2% starch, 0.11% calcium carbonate, pH

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Fig. 1. Structure of GE2270.



The numbering of rings and atoms is indicated.

7.4) added with 50  $\mu\text{g/ml}$  vitamin B12. Routinely, the strain was grown for 72 hours in vegetative medium before using a 5% inoculum into production medium. All cultures were grown at 28°C with shaking.

All labelled precursors were purchased from Cambridge Isotope Laboratories, except for [2- $^{13}\text{C}$ ]glycine which was from Sigma Aldrich, and were at a  $^{13}\text{C}$  purity of 99%. The amount of GE2270 produced under different conditions was evaluated on a Varian HPLC system equipped with a 5  $\mu\text{m}$   $\text{C}_{18}$  column (Beckman). The antibiotic was eluted with a gradient of 2 g/liter ammonium formate- $\text{CH}_3\text{CN}$ . Before NMR analysis, the antibiotic was recovered from the pelleted mycelium by extraction twice with 1 vol of  $\text{CH}_3\text{CN}:\text{H}_2\text{O}$  7:3. The dried extract was then purified by preparative TLC developed with  $\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}$  93:7, eluted with  $\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}$  7:3 and lyophilized in deuterated DMSO.

Spectra of GE2270 (13 to 21 mg/ml in  $\text{DMSO-}d_6$ ) were obtained with a Bruker DRX 500 Fourier transform spectrometer under the following conditions: frequency 125.77 MHz, acquisition time 0.7 seconds, 32K of memory, 11,000 transients, spectral width 26,881 Hz, flip angle 90°, internal reference  $\text{DMSO-}d_6$   $\delta=39.43$  ppm, temperature 40°C. Broadband (1H)-decoupling was achieved by inverse gated sequence. The FIDs were processed with 2 Hz of

Lorentzian line broadening. The distribution of isotopic label was determined by comparing  $^{13}\text{C}$  NMR spectra of the enriched and natural abundance compounds recorded and processed under identical experimental conditions. Percent incorporations were determined as ratio  $r$ , for each peak. The methyl resonance of valine (17.8 ppm) was used as internal reference according to the following formula:

$$r = \frac{R_L}{R_N} \times \frac{P_N}{P_L}$$

where  $R_L$  is the intensity of the labelled peak,  $R_N$  is the intensity of the peak in the natural abundance spectrum,  $P_N$  and  $P_L$  are the intensities of the valine methyl in natural abundance and labelled spectrum, respectively. Assignments of the various carbons were as in ref. 17, except for those of C-4 and C-5 from thiazole D (see below).

## Results and Discussion

Under the experimental conditions described in Materials and Methods, the amount of GE2270 produced by the *P. rosea* strain employed in this study varied between 300 and 500  $\mu\text{g/ml}$  after 68–120 hours of growth in production medium. Before adding the labelled precursors, GE2270 production was evaluated upon addition of the unlabeled compounds. In 100-ml production medium, the addition of two aliquot of up to 300 mg each of acetate (after 9 and 16 hours of growth), or of up to 30 mg each of DL-serine (after 24 and 48 hours of growth), had little effect on GE2270 titers. However, addition (after 24 and 48 hours) of two 20-mg aliquots of glycine severely reduced GE2270 levels to ca. 100  $\mu\text{g/ml}$ . Nonetheless, these titers were sufficient to recover enough antibiotic for NMR analysis.

A labelling experiment was performed adding 10 mg of [2- $^{13}\text{C}$ ]glycine after 24 and 40 hours of growth in the production medium. The total isotopic enrichment, evaluated by MS analysis, was about 50%. The observed specific enrichments have been ascribed to: the  $\alpha$ -carbon of the glycine residue; to the C-4 positions of the six thiazole rings; to the C-5 positions of thiazole rings C, D and E; to the C-methyl(ene) groups of thiazoles D and E; to the N- and O-methyl groups of thiazole D and of the Asn residue, respectively; to the C-4 and C-5 carbons of the oxazoline ring; to the C-3, C-4 and C-5 carbons of the pyridine ring (see Table 1 and Fig. 2). One enrichment was also observed at 150.0 ppm, a position corresponding to either C-2 or C-6 of the pyridine ring. By analogy with other thiazolylpeptides (see below), this enrichment is likely due to C-6. [It should

be noted that the signals at 140.7 and 143.3 ppm were originally attributed to C-5 and C-4 positions of thiazole D, respectively. The results from the [2-<sup>13</sup>C]-glycine labelling and those from [3-<sup>13</sup>C]-serine (see below) are consistent with a labelling pattern whereby the C-4 position of each thiazole is labelled by [C-2]-glycine. This indicates that the original assignments for C-4 and C-5 of thiazole D were probably inverted, so that thiazole D represents an exception with respect to the other thiazole, in having the C-4 resonance at slightly lower fields than C-5 (see Table 1).]

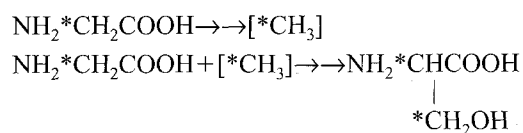
A labelling experiment, performed adding 20 mg of L-[3-<sup>13</sup>C]-serine after 24 and 48 hours of growth in the production medium, gave a total isotopic enrichment of about 25%. The specific enrichments observed in this case are reported in Table 1. These signals have been ascribed to the C-5 positions of the six thiazoles; to the C-methyl(ene) groups of thiazoles D and E; to the *N*- and *O*-methyl groups; to the C-5 carbon of the oxazoline; and to the C-4 and C-5 carbons of the pyridine ring. The results of the feeding experiments with Gly and Ser are summarized in Fig. 2. They indicate that the C-4 position of all thiazoles is labelled by C-2 of glycine, while the C-5 position is labelled by C-3 of serine. Thus, formation of GE2270 is very similar to that of other thiazolylpeptides: the thiazole rings are derived from serine, presumably after its conversion to cysteine; the pyridine ring is derived from the head to head condensation of two serine residues; and the oxazoline results from cyclization of serine.

These results were confirmed by a labelling experiment with [U-<sup>13</sup>C]-glycine, added under the same conditions as [2-<sup>13</sup>C]-glycine. Satellite resonances, due to <sup>13</sup>C-<sup>13</sup>C spin-spin coupling, indicate incorporation of an intact glycine residue. This was observed, besides for the glycine residue itself, for the oxazoline, for all the thiazoles and for the pyridine ring (two units). Thus, a total ten intact glycine residues are incorporated into GE2270. These results, summarized in Fig. 2, are consistent with the enrichments from [2-<sup>13</sup>C]-glycine and [3-<sup>13</sup>C]-serine, and confirm the hypothesis that glycine is converted into serine, which in turn is the source of oxazoline rings and, presumably after its conversion into cysteine, of the thiazoles. The percentage enrichments after labeling with [U-<sup>13</sup>C]-glycine were also evaluated, considering the sum of the intensities of the central peak and of its satellites without any correction. Due to this fact and to the frequent overlapping of resonances, the ratio *r* obtained for some positions of the [U-<sup>13</sup>C]-glycine-fed GE2270 should be considered approximate. Most of the carbon atoms that were labelled either by [2-<sup>13</sup>C]-glycine or by [3-<sup>13</sup>C]-serine were also

enriched by [U-<sup>13</sup>C]-glycine. The only exceptions were the C-5 positions of thiazoles A and F (Table 1). Furthermore, additional enrichments were observed at the C' positions of thiazoles D, E and F, and of the oxazoline; at C-2 of thiazoles A, B and F; and at C-2 of the oxazoline. Thus, apart from the carbons of the Asn, Val, ( $\beta$ -OH)-Phe and Pro residues, most positions in GE2270 showed various degrees of enrichment.

It should be noted that the best enrichments for [2-<sup>13</sup>C]-glycine were observed at positions corresponding to either C-2 of the Gly residue, or to C-2 of Ser-derived residues, indicating an efficient conversion of Gly into Ser. Furthermore, significant enrichments were also seen at positions equivalent to C-3 of serine-derived residues, and to the *C*-, *N*- and *O*-methyls, indicating that C-2 of glycine can be an efficient source of methyl equivalents. Also [3-<sup>13</sup>C]-serine was an efficient source of methyl groups (Table 1). The enrichment observed for the two *C*-methyl groups was equivalent to that seen for the *N*- and *O*-methyls, indicating that all these methyls are derived from a cellular methyl donor. Thus, our results are consistent with thiazoles D and E being derived from cysteine, and subsequently being converted to the *C*-methylated forms. We tried also a labelling experiment with threonine. However, this amino acid severely repressed synthesis of GE2270. Consequently, we were unable to isolate enough labelled antibiotic for NMR analysis. Nonetheless, our results make it unlikely that the *C*-methylated thiazoles derive from incorporation of an *S*-analog of threonine. The observation that the methylene group of glycine is an effective source of methylating equivalents has been made during sulfomycin biosynthesis by *Streptomyces arginensis*<sup>12)</sup>, although in other studies of thiazolylpeptide formation by other actinomycete strains this phenomenon was not observed<sup>8)</sup>.

Addition of [2-<sup>13</sup>C]-glycine resulted in enrichment of C-4 and C-5 of the oxazoline, as well as C-2 through C-5 of the pyridine ring. This result is consistent with the existence of a pathway whereby glycine is converted to serine by methylation, and C-2 of glycine can generate methyl groups, as outlined below:



Judging from the similar enrichments seen for C-2 of the Gly residue and for C- $\alpha$  of the oxazoline, it appears that the conversion of glycine into serine is quite efficient. On the other hand, the substantially higher enrichment seen at the oxazoline than at the thiazoles after feeding [3-<sup>13</sup>C]-serine,

Table 1. Incorporation ratios for the enrichment of GE2270 with  $^{13}\text{C}$ -labeled precursors.

Relative  $^{13}\text{C}$ -enrichment (enriched/natural)

Carbon assignment	$^{13}\text{C}$ $\delta$ (ppm)	[ $2\text{-}^{13}\text{C}$ ]-Glycine	DL-[ $3\text{-}^{13}\text{C}$ ]-Serine	[ $1,2\text{-}^{13}\text{C}$ ]-Glycine	[ $2\text{-}^{13}\text{C}$ ]-acetate
CH <sub>3</sub> Thiaz.E	11.62	5.8	7.0	6.1	
N-CH <sub>3</sub> Asn	25.45	5.6	6.5	6.0	
$\beta$ Asn	37.32				3.6
$\alpha$ Gly	40.89	8.9		6.6	
$\alpha$ Asn	47.84				3.7
CH <sub>2</sub> Thiaz.D	58.25	6.2	7.8	6.1	
OCH <sub>3</sub> Thiaz.D	67.00	5.9	7.2	6.2	
C-4 Oxazoline	67.77	9.5		8.2	
C-5 Oxazoline	69.05	4.7	9.5	5.7	
C-5 Thiaz.C	116.0	1.7	3.7	1.5	
C-5 Pyridine	118.2	5.6	9.9	3.2	
C-5 Thiaz.B	122.7		4.9	1.6	
C-5 Thiaz.F	126.5		4.2		
C-3 Pyridine	127.4	7.8		5.9	
C-5 Thiaz.A	128.3		4.3		
C-5 Thiaz.E	139.1	1.5	3.2		
C-4 Thiaz.D <sup>a</sup>	140.7	2.6		1.6	
C-4 Pyridine	140.9	4.4	3.6	5.3	
C-4 Thiaz.E	141.7	2.6		2.9	
C-5 Thiaz.D <sup>a</sup>	143.3	1.5	3.0	1.5	
C-4 Thiaz.A	144.4	2.5		2.2	
C-4 Thiaz.C	146.5	2.8		2.4	
C-4 Thiaz.F	149.1	2.5		2.7	
C-2 or C-6 Pyr	150.0	7.9		6.6	
C-4 Thiaz.B	153.1	2.0		2.0	
C-2 Oxazoline	158.9			2.0	
C' Thiaz.F	159.9			1.9	
C-2 Thiaz.B	160.2			2.4	
C' Thiaz.E	160.8			2.5	
C-2 Thiaz.F	164.4			6.5	1.7
C-2 Thiaz.D	165.3				1.4
C'-Oxazoline	167.4			6.6	1.7
C-2 Thiaz.A	167.9			5.8	1.7
C-2 Thiaz.E	168.0				2.4
C' Gly	169.2	6.6			1.7
$\gamma$ Asn	169.4				2.7
C' Pro	173.3				1.3

<sup>a</sup>original assignments modified after this work.

suggests a substantial dilution of the label prior to incorporation into the thiazoles. It remains to be determined whether this apparent dilution is due to an excess of unlabelled precursor of the thiazoles (presumably cysteine)

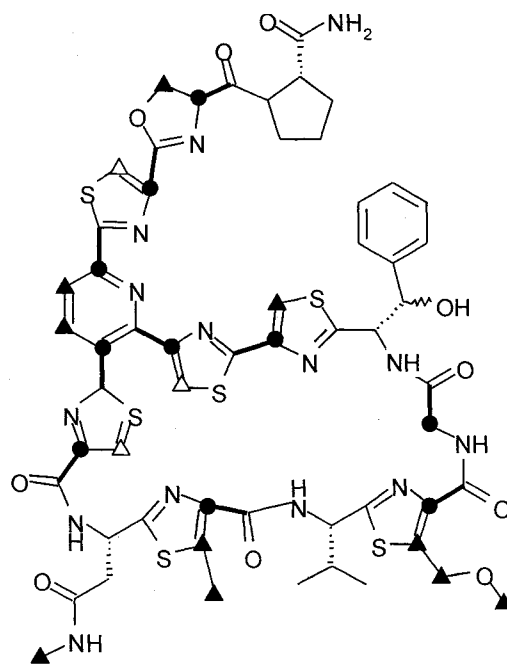
available for GE2270 formation, or to an inefficient conversion of serine into this precursor. The enrichments at C-5 of thiazoles after feeding [ $2\text{-}^{13}\text{C}$ ]-glycine are consistent with this label dilution: only three of the C-5 positions of

thiazole rings gave a significant enrichment, whereas for the other three the enrichment levels were very close to background, indicating a processive dilution as glycine is first converted into serine and the latter into cysteine. An alternative explanation for this observation might be due to the lower titers of GE2270 observed after addition of glycine (about 30% of control). Thus, the efficient conversion of glycine into serine might result from a repression or inhibition of serine utilization by glycine.

It is interesting to observe that, under our experimental conditions, the conversion of glycine to serine appears to be equivalent to the conversion of serine to cysteine, as judged from the similar enrichments observed at C-4 of the oxazoline when feeding [2-<sup>13</sup>C]-glycine, to that of C-5 of thiazoles, when feeding [3-<sup>13</sup>C]-serine. One anomaly that we observed in the pyridine enrichments is the higher level of enrichment observed at C-4 compared to that of C-5 after feeding [3-<sup>13</sup>C]-serine. It has been proposed that the pyridine ring results from head to head condensation of two serine residues<sup>13</sup>. The synthesis of the peptide chain in a thiazolypeptide is believed to start from the pyridine ring, thereby the C-4 position of pyridine would correspond to C-3 of a putative starting (amino) acid. The lower enrichment at C-4 than at C-5 is consistent with the hypothesis that this putative initiator may not be serine itself, but one of its metabolites. It is worth mentioning that also MOCEK *et al.* reported a similar discrepancy in the relative enrichments of the pyridine ring during their studies on the biosynthesis of thiostrepton<sup>10</sup>.

The origin of the other residues in GE2270 has not been directly investigated. However, addition of [2-<sup>13</sup>C]-acetate yielded some modest but significant enrichments (Table 1). The strongest enrichment was observed for all the carbons of the Asn residue and, to a lesser extent, for the C-2 positions of thiazole ring E. Smaller but significant enrichments were also observed at the C-2 positions of thiazoles A, D and F, as well as at the C' positions of oxazoline and glycine (see Fig. 2). As expected by analogy with other thiazolypeptides, acetate is poorly utilized as a carbon source during GE2270 formation under the experimental conditions employed. However, the labelling pattern observed for the asparagine residue is consistent with that expected after entry of acetate into the Krebs cycle and its conversion into oxaloacetate, which would then be transaminated to asparagine. The weak enrichment (but significantly above background) for the C-1 position of glycine from C-2 of acetate is consistent with similar enrichments seen at C-2 of at least some of the thiazoles. The subsequent conversion of glycine into serine by methylation would be consistent with the labelling of the C-

Fig. 2. Labeled positions in GE2270.



Positions labeled by [2-<sup>13</sup>C]-glycine are indicated by a dot; those labeled by [2-<sup>13</sup>C]-glycine and [3-<sup>13</sup>C]-serine by a filled triangle; those labeled by [3-<sup>13</sup>C]-serine only by an empty triangle. Incorporation of an intact glycine residue is indicated by a thick line.

2 carbons of some thiazoles. Thus, our results suggest the existence of a pathway whereby acetate is converted into glycine in such a way that C-2 of acetate would become C-1 but not C-2 of glycine. We are unaware of reports describing such a conversion of acetate into glycine in actinomycetes.

In conclusion, our results show that the biosynthetic route to GE2270 can be considered very similar to that of better studied thiazolypeptides such as nosiheptide and thiostrepton<sup>9,10</sup>. They also point to the existence in *P. rosea* of an active pathway for generating methylating equivalents from C-2 of glycine. In addition, it appears that glycine is synthesized in this strain by a pathway involving the conversion of C-2 of acetate into C-1 of glycine.

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