Journal of Industrial Microbiology, 1 (1987) 341–347
Elsevier

SIM 00046

Deacetylation of *N*-acetylthienamycin to thienamycin by a cell-free extract of *Streptomyces cattleya*, the thienamycin producer

Masaru Uyeda* and Arnold L. Demain

Fermentation Microbiology Laboratory, Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.

Received 21 July 1986
Revised 20 November 1986
Accepted 20 November 1986

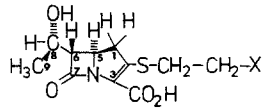
Key words: Antibiotic biosynthesis; Beta-lactam antibiotic; Carbapenem antibiotic

SUMMARY

A cell-free extract from the thienamycin producer, *Streptomyces cattleya*, has been found to deacetylate the co-product, *N*-acetylthienamycin. The pH optimum of the reaction is 7.5. Due to the lability of *N*-acetylthienamycin, we used the D and L forms of the synthetic substrate *N*-chloroacetylvaline. We found that the enzyme is an L-deacetylase, has a molecular weight of 58 000, is stable up to 40°C, acts optimally at 45°C, is stable at pH 5–8, is not activated by divalent metal ions and is inhibited by Hg^{++}, Cu^{++} and *p*-chloromercuribenzoate. This is the first report of an extract from a carbapenem producer which carries out the deacetylation of *N*-acetylthienamycin, suggesting that the acetylated derivative is a precursor of thienamycin.

INTRODUCTION

Thienamycin (Fig. 1) was the first of a series of naturally occurring β-lactam antibiotics possessing the carbapenem ring to be discovered [8]. Biosynthesis of the antibiotic in intact cells of *Streptomyces cattleya* has been studied by Williamson et al. [13]. We have been attempting to demonstrate some of the reactions of the biosynthetic pathway with cell-free extracts. Since *S. cattleya* produces *N*-acetylthienamycin along with THM [8], we considered the possibility that the *N*-acetylated com-



X = NH_2, Thienamycin
X = $NH-OC-CH_3$, N-Acetyl thienamycin

Fig. 1. Structure of thienamycin and *N*-acetylthienamycin.

* Present address: Laboratory of Medicinal Microbiology, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan.

Abbreviations: THM, thienamycin; N-AcTHM, *N*-acetylthienamycin; CFE, cell-free extract; N-Cl-Ac-L-Val, *N*-chloroacetyl-L-valine; N-Cl-Ac-D-Val, *N*-chloroacetyl-D-valine.

Correspondence: Dr. A.L. Demain, Fermentation Microbiology Laboratory, Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

pound is a precursor. We now report that a cell-free extract catalyzes the conversion of N-AcTHM to THM.

MATERIALS AND METHODS

Culture

The strain of *S. cattleya* used was NRRL 8057, the original soil isolate (MA-4297) [8]. The culture, as well as the antibiotics, THM and N-AcTHM, were supplied by Merck & Co. Inc. The other *N*-acetylated carbapenem antibiotic, PS-5, was obtained from the Sanraku Co.

Media

The complex seed medium (sucrose, solulac, ardamine YEP and corn gluten meal), semi-synthetic seed medium (glycerol, glutamate, ardamine YEP, inositol, *p*-aminobenzoic acid, NH_4Cl and other mineral salts), and synthetic production medium A (glycerol, glutamate, isoleucine, MES buffer, NH_4Cl and other mineral salts) were the same as previously described [13].

Preparation of resting cells

Resting cells were prepared as previously described [13] and stored at 4°C until used.

Preparation of cell-free extract

The resting cell suspension was sonicated on ice with a Branson cell disruptor (model 200) under the following conditions: output-setting of 5, 50% duty cycle intermittently for 2–3 min (actual sonic exposure, 1–1.5 min). The suspension was centrifuged at 12 000 × *g* for 15 min to provide a cell-free extract. In some cases, the cell-free extract was dialyzed against 0.02 M MES buffer (pH 7.0).

Conversion of N-AcTHM to THM by deacetylation

The incubation mixture contained the following in a total volume of 0.1 ml: 16 mM N-AcTHM, 40 mM K-phosphate buffer (pH 7.5) and 50 μl cell-free extract containing about 250 μg protein. After incubation for 0, 1, 3, 5 h at 30°C, the reaction products were chromatographed on a cellulose TLC plate (Eastman chromatogram sheet, 13255, cellulose) using ethanol/water (7:3) as solvent. Estimation of the conversion was done by bioautography with *Staphylococcus aureus* ATCC 6538P. A sterile paper tissue was placed over seeded agar in a Petri dish and the TLC plate was placed face down on the paper for 20 min. The paper and the TLC plate were removed and the dish was incubated overnight at 37°C.

Assay of deacetylase activity

N-Chloroacetyl-L-valine and *N*-chloroacetyl-D-valine (Sigma Chemical Co., St. Louis, MO) were used to measure L-amino acid and D-amino acid deacetylase activities, respectively. After dissolving the substrates in distilled water, the solutions were adjusted to pH 7.5 with 1 N NaOH. The reaction mixture in a final volume of 50 μl contained 1 μmol of substrate, 2.5 μmol of K-phosphate buffer (pH 7.5) and 10–20 μl of enzyme containing about 100 μg protein. Incubation was for 5–30 min at 30°C. The mixture was cooled immediately on ice and 50 μl of 50% acetic acid were added to terminate the reaction. The ninhydrin method of Yemm and Cocking [15] was used to determine the valine released. The molar extinction of valine is 21.4 at 570 nm. One unit of activity is that which releases 1 μmol of valine per h. Specific activities are expressed as units per mg protein. Protein was estimated after addition of 5-fold-diluted Bio-Rad dye concentrate (Bio-Rad Laboratories, Richmond, CA) by the method of Bradford [1].

Unless otherwise stated, all steps of purification were carried out in a cold room at 5°C. Potassium phosphate buffer, pH 7.5, was used throughout. To remove nucleic acid, 50% streptomycin sulfate was added dropwise with gentle stirring to the cell-free extract to give a final concentration of 0.4% streptomycin sulfate. The solution was stirred for a further 30 min and centrifuged to yield a clear enzyme solution. Then, ammonium sulfate was slowly added with agitation to 60% saturation. After stirring for a further 30 min, the precipitate was recovered by centrifugation. It was dissolved in a small amount of the buffer (0.02 M) and dialyzed twice against the same buffer. The active solution,

thus obtained, was applied to a DEAE-Sephacel column (2.4 × 14 cm) that had been equilibrated with 0.02 M buffer. After being washed with the same buffer, the enzyme activity was eluted with a linear gradient concentration of KCl from 0 to 1 M in the same buffer. The active enzyme fractions were pooled, concentrated by salting-out with ammonium sulfate, and filtered through a Sephadex G-100 column (1.5 × 45 cm) that had been equilibrated with 0.02 M buffer. The active enzyme fractions were pooled and passed through the same column after concentration.

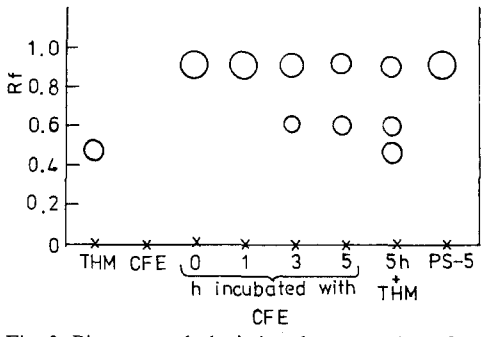


Fig. 3. Bioautograph depicting the conversion of PS-5 to NS-5 by the *S. cattleya* extract. Although authentic NS-5 was not available, the spot at R_f 0.6 is known to be NS-5 (see text).

RESULTS

Conversion of N-AcTHM to THM by the cell-free extract of S. cattleya

A bioautogram illustrating the conversion of N-AcTHM to THM is diagrammatically shown in Fig. 2. N-AcTHM was deacetylated to THM by the cell-free extract but not by a boiled (15 min) cell-free extract. We failed to detect acetylation of THM to N-AcTHM.

Conversion of PS-5 to NS-5 by the same cell-free extract

As shown in Fig. 3, PS-5, another *N*-acetylated carbapenem antibiotic, was deacetylated by the same cell-free extract to form NS-5 (deacetylated PS-5). NS-5 was identified by its R_f value of 0.22 in paper chromatography using a solvent system of acetonitrile/0.1 M Tris-HCl (pH 7.5)/0.1 M EDTA, pH 7.5 (120:30:1) [2] after isolation of this compound from the reaction mixture by preparative paper chromatography.

Effect of pH on the conversion of N-AcTHM to THM

The effect of pH on the conversion of N-AcTHM to THM was examined in the pH range from 5 to 9.2. The buffers used were K-phosphate (pH 5–8) and sodium borate (pH 9.2). From the measurement of the diameters of the inhibitory zones corresponding to THM, antibacterial activity was estimated semi-quantitatively after bioautography. As shown in Fig. 4, the conversion occurred most effectively at pH 7.5.

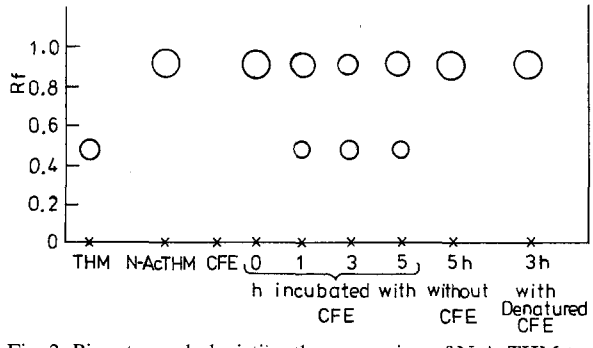


Fig. 2. Bioautograph depicting the conversion of N-AcTHM to THM by the cell-free extract.

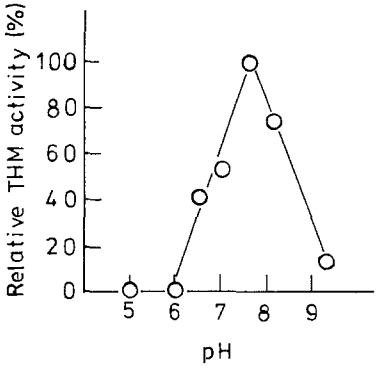


Fig. 4. Effect of pH on the conversion of N-AcTHM to THM. Activity is shown as percent of maximum rate observed.

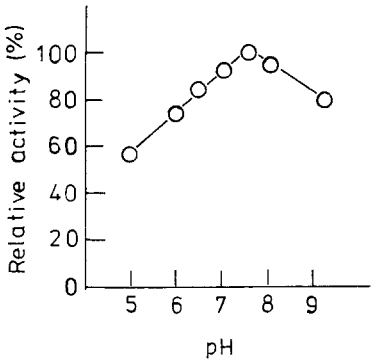


Fig. 5. Effect of pH on the deacetylation of *N*-chloroacetyl-L-valine. Activity is shown as percent of maximum rate observed.

Optimum reaction pH of deacetylase on a synthetic substrate

The optimal pH value for deacetylation of *N*-chloroacetyl-L-valine was also 7.5 (Fig. 5).

Since THM and other carbapenem antibiotics are fairly labile under physiological conditions, their quantitative assay is very difficult. In addition, THM is more unstable than N-AcTHM because of its free amino group; the same is true for PS-5 and NS-5 [2]. For these reasons, we decided to use *N*-chloroacetyl-L-valine and *N*-chloroacetyl-D-valine [3] for further studies on the deacetylase activity of the *S. cattleya* cell-free extract.

Stereospecificity of the deacetylase activity

When *N*-chloroacetyl-L-valine and *N*-chloroacetyl-D-valine were used as substrates for the cell-free extract from *S. cattleya*, the activity was found to be mainly that of an L-deacetylase. D-Deacetylase activity was found to be negligibly small (only 1–3% of the L-enzyme activity; Table 1). Therefore, only the L-amino acid deacetylase activity was examined in the remaining experiments.

Table 1

Deacetylase activity in the cell-free extract from *S. cattleya*

Enzyme source	OD at 570 nm					
Substrate:	N-Cl-Ac-L-Val			N-Cl-Ac-D-Val		
	0 min	30 min	Δ	0 min	30 min	Δ
Cell-free extract No. 1	0.17	1.02	0.85	0.22	0.24	0.02
Cell-free extract No. 2	0.24	0.89	0.65	0.23	0.24	0.01
Cell-free extract No. 3	0.06	0.82	0.76	0.06	0.08	0.02

Partial purification of L-*amino acid deacetylase*

The L-amino acid deacetylase was partially purified from the cell-free extract of *S. cattleya* by the procedure described in Materials and Methods. Table 2 summarizes the results. The enzyme was purified about 19-fold from the cell-free extract. Caseinolytic activity was not observed with this partially purified preparation. The partially purified enzyme was able to convert N-AcTHM to THM.

Table 2

Summary of purification of *S. cattleya* L-deacetylase

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield of activity (%)	Degree of purification
Cell-free extract	13 236	267.5	49.5	100	1.0
Streptomycin treatment	11 938	329.6	36.2	90.2	0.7
$(NH_4)_2SO_4$-salting out	7 550	79.1	95.4	57.0	1.9
DEAE-Sephacel	4 778	24.9	191.9	36.1	3.9
Sephadex G-100	2 778	5.2	534.2	21.0	10.8
Retreatment with G-100	1 271	1.4	907.8	9.6	18.7

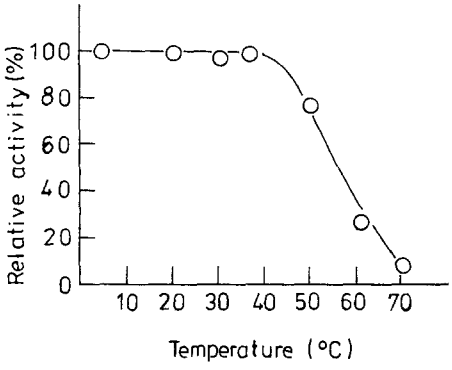


Fig. 6. Effect of temperature on the stability of the L-amino acid deacetylase. The enzyme was preincubated for 15 min at the temperature depicted before assaying the residual activity. Activity is shown as percent of maximum rate observed.

Using the partially purified preparation, characteristics of the enzyme were examined. Results are described in the following paragraphs.

Molecular weight

By comparing the elution of the enzyme from Sephadex G-100 with that of standard protein markers, the molecular weight of the deacetylase was estimated to be 58 000. The standard proteins used were trypsin (24 000) ovalbumin (46 000) and bovine serum albumin (67 000).

Thermostability

Fig. 6 shows the enzyme to be stable at temperatures of up to about 40°C.

Optimum reaction temperature

The effect of the incubation temperature on the reaction velocity is shown in Fig. 7. The optimum reaction temperature is 45°C.

pH stability

To determine pH stability, the enzyme was kept at 4°C for 24 h in 25 mM sodium citrate buffer (pH 4), K-phosphate buffer (pH 5–8) or sodium borate buffer (pH 9). The assay was done in 100 mM K-phosphate buffer at pH 7.5. As shown in Fig. 8, the enzyme was stable over the range of pH 5–8.

Effect of divalent metal ions

It is well known that L-amino acid acylases are activated by Co^{++} [3]. The effects of divalent metal ions were examined on the *S. cattleya* deacetylase acting on *N*-chloroacetyl-L-valine. As shown in Table 3, the enzyme was not activated by any divalent metal ion. Hg^{++} and Cu^{++} ions strongly inhibited the deacetylase.

Effect of inhibitors

The effect of some enzyme inhibitors on the deacetylase activity was examined as shown in Table 4. The enzyme was strongly inhibited by 1 mM *p*-chloromercuribenzoate but not by EDTA.

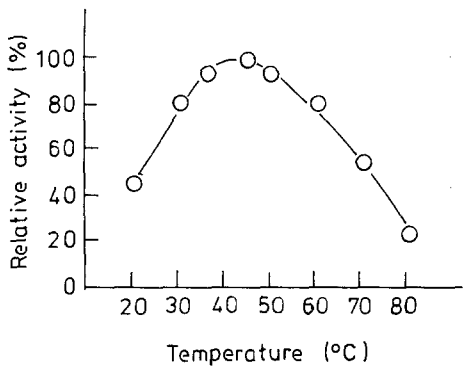


Fig. 7. Effect of temperature on activity of the L-amino acid deacetylase. Activity is shown as percent of maximum rate observed.

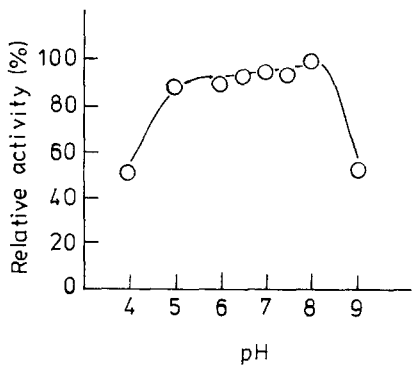


Fig. 8. Effect of pH on the stability of the L-amino acid deacetylase. Activity is shown as percent of maximum rate observed.

Table 3

Effect of divalent metal ions on the deacetylase activity

The metal ions were added to the standard assay mixture using *N*-chloroacetyl-L-valine as substrate.

Metal ion	Concn. (mM)	Relative activity (%)
None		100
$HgCl_2$	0.1	1
$CuSO_4$	0.1	50
$MnCl_2$	1	98
$ZnSO_4$	1	94
$CdSO_4$	1	101
Pb-acetate	1	105
$MgSO_4$	1	105
$FeSO_4$	1	111
$NiSO_4$	1	77
$CoCl_2$	0.1	100
$CoCl_2$	1	98
$CoCl_2$	4	103

DISCUSSION

The carbapenem antibiotics are among the most potent, broad-spectrum and non-toxic antibacterial agents ever discovered. Of this group, THM is the most active and as its semi-synthetic derivative, imipenem (*N*-formimidoylthienamycin; MK-787), is the only one being used in medicine. Although a considerable amount is known about THM precursors from studies on intact cells [13,14], no cell-free studies have been reported yet on *S. cattleya*. Such cell-free systems are needed to elucidate the exact pathway of thienamycin biosynthesis and to study the enzymes involved.

Table 4

Effect of inhibitors on L-amino acid deacetylase

The enzyme was pre-incubated with 1 mM of the inhibitors for 10 min at 30°C before assay.

Inhibitor	Relative activity (%)
None	100
NaN_3	91
PMSF	109
p-CMB	20
EDTA	98
KCN	105
N-Cl-Ac-D-Val	103

This paper reports on an enzyme in *S. cattleya* that deacetylates N-AcTHM to THM. THM is several times more active than N-AcTHM in its antibacterial activity. Since both compounds are produced by *S. cattleya*, it is reasonable to postulate that the L-amino acid deacetylase described in this work is a biosynthetic enzyme, possibly the last in the sequence, involved in THM formation.

Other enzymes have been described which deacetylate THM or PS-5. Kahan and Kahan [4–7] have disclosed in Japanese patents that an enzyme from *Escherichia coli* can deacylate and acylate compounds of the THM group and that *N*-acetylethanolamine amidohydrolase from *Protaminobacter ruber* can deacylate THM-related compounds. We have failed to observe acetylation of THM with our *S. cattleya* extract. Fukagawa and coworkers [2,9] have described enzymes from *Pseudomonas* strain 1158, *Streptomyces olivaceus* and porcine kidney which deacylate PS-5 to NS-5. However, in none of these cases is the enzyme from a source which is known to produce carbapenem antibiotics. The pseudomonad produces two deacylating enzymes, L-amino acid acylase and D-amino acid acylase in a ratio of 3 to 1; both attacked PS-5. The activity of the partially purified enzyme preparation from *S. cattleya* is mainly active on substrates of the L-configuration and differs from the *Pseudomonas* enzyme [10] in molecular weight, pH and temperature optima, pH stability, cobalt activation and EDTA inhibition.

The *S. cattleya* deacetylating activity is probably a sulfhydryl enzyme as evidenced by its susceptibility to inhibition by *p*-chloromercuribenzoate, Hg^{++} and Cu^{++} and its tolerance to EDTA.

A different type of deacylase [11] has been obtained from the carbapenem producer *Streptomyces fulvoviridis* A933, which forms the following carbapenems: PS-5, epithienamycins A and C, and MM 17880. Certain mutants of this culture form

the OA-6129 series which contains a pantothenyl group attached to the cysteaminyl side-chain. This deacylase (called A933 acylase) attacks *N*-acetylated L-amino acids and some L-dipeptides, and removes the panthothenyl side-chain of the OA-6129 type carbapenems. However, since the A933 acylase does not deacetylate PS-5, it is clearly different from our L-deacetylase. Furthermore, the A933 acylase has a higher molecular weight (ca. 100 000) and, unlike the *S. cattleya* enzyme, is inhibited by Zn^{++}, Fe^{++}, Cd^{++}, Co^{++} and Pb^{++}. It would appear that the A933 acylase functions in the conversion of the OA-6129 series to the non-pantothenyl carbapenems whereas our deacylase acts to convert N-AcTHM to THM. The isolation [12] of a *S. cattleya* mutant which produces N-AcTHM but not THM supports the latter hypothesis.

ACKNOWLEDGEMENTS

We thank Barbara Lago and Edward Inamine of the Merck Sharp & Dohme Research Laboratories for *S. cattleya* MA4297 (NRRL 8057), *Staphylococcus aureus* ATCC 6538P, THM, N-AcTHM and constituents of culture media. We also thank T. Ishikura of Sanraku Co. for PS-5. M. Uyeda was supported by an Overseas Research Fellowship from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

2 Fukagawa, Y., K. Kubo, T. Ishikura and K. Kouno. 1980. Deacetylation of PS-5, a new β-lactam compound. I. Microbial deacetylation of PS-5. J. Antibiot. 33: 543–549.

3 Greenstein, J.P. and M. Winitz. 1961. Chemistry of the Amino Acids. Vol. 2, p. 1753. John Wiley and Sons, Inc., New York.

4 Kahan, J.S. and F.M. Kahan. 1977. Antibiotics and their preparation. Japan Kokai 79-65,295.

5 Kahan, J.S. and F.M. Kahan. 1978. Antibiotic desacetyldihydro $890A_9$. Japan Kokai 78-103,494.

6 Kahan, J.S. and F.M. Kahan. 1979. Enzymatic hydrolysis of N-acylthienamycin. Japan Kokai 79-46,890.

7 Kahan, J.S. and F.M. Kahan. 1979. Method for preparation of N-acylthienamycin. Japan Kokai 79–55,789.

8 Kahan, J.S., F.M. Kahan, R. Geogelman, S.A. Currie, M. Jackson, E.O. Stapley, T.W. Miller, A.K. Miller, D. Hendlin, S. Mochales, S. Hernandez, H.B. Woodruff and J. Birnbaum. 1979. Thienamycin, a new β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J. Antibiot. 32: 1–12.

9 Kubo, K., T. Ishikura and Y. Fukagawa. 1980. Deacetylation of PS-5, a new β-lactam compound. II. Separation and purification of L- and D-amino acid acylases from *Pseudomonas* sp. 1158. J. Antibiot. 33: 550–555.

10 Kubo, K., T. Ishikura and Y. Fukagawa. 1980. Deacetylation of PS-5, a new β-lactam compound. III. Enzymological characterization of L-amino acid acylase and D-amino acid acylase from *Pseudomonas* sp. 1158. J. Antibiot. 33: 556–565.

11 Kubo, K., T. Ishikura and Y. Fukagawa. 1985. Studies on the biosynthesis of carbapenem antibioptics. 3. Enzymological characterization of the L-amino acid acylase activity of A933 acylase. J. Antibiot. 38: 622–630.

12 Rosi, D., M.L. Drozd, M.F., Kuhrt, L. Terminiello, P.E. Came and S.J. Daum. 1981. Mutants of *Streptomyces cattleya* producing N-acetyl and deshydroxy carbapenems related to thienamycin. J. Antibiot. 34: 341–343.

13 Williamson, J.M., E. Inamine, K.E. Wilson, A.W. Douglas, J.M. Liesch and G. Albers-Schonberg. 1985. Biosynthesis of the β-lactam antibiotic thienamycin, by *Streptomyces cattleya*. J. Biol. Chem. 260: 4637–4647.

14 Williamson, J.M., R. Meyer and E. Inamine. 1985. Reverse transsulfuration and its relationship to thienamycin biosynthesis in *Streptomyces cattleya*. Antimicrob. Agents Chemother. 28: 478–484.

15 Yemm, E.W. and E.C. Cocking. 1955. Determination of amino acids with ninhydrin. Analyst 80: 209–213.