THE BIOSYNTHETIC IMPLICATIONS OF ACETATE AND GLUTAMATE INCORPORATION INTO (3*R*,5*R*)-CARBAPENAM-3-CARBOXYLIC ACID AND (5*R*)-CARBAPEN-2-EM-3-CARBOXYLIC ACID BY *SERRATIA* SP.

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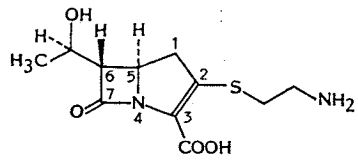
(Received for publication February 17, 1988)

Two new β-lactams have been isolated from strains of *Serratia* and *Erwinia* sp. and identified as (3*R*,5*R*)- and (3*S*,5*R*)-carbapenam-3-carboxylic acid. These novel carbapenams lack antibacterial activity, are resistant to both β-lactamases I and II from *Bacillus cereus* and are not detected by the lactamase induction assay. Radiolabelled and stable isotope experiments have established that both metabolites together with the antibiotic 5*R*-carbapenem-3-carboxylic acid are glutamate and acetate derived. A number of possible pathways for the biosynthesis of these compounds as well as their relationship to the more complex members of the carbapenem family of β-lactam antibiotics are discussed.

The β-lactam antibiotics thienamycins (**1**) and the olivanic acids (**2**) which possess the carbapenem ring system are now representative of a substantial family of naturally occurring compounds[1~4]. These have mainly been isolated from *Streptomyces* species and to date biosynthetic studies on this important group of antibiotics have been restricted by the complexity, low yield and relative instability of the carbapenems produced. Radioactive and stable isotope studies with *Streptomyces cattleya* have demonstrated that, in thienamycin (**1**), the C_6 and C_7 of the β-lactam ring are derived from acetate, the cysteaminyl side-chain from cysteine and both carbon atoms of the hydroxyethyl substituent at C_6 from the methyl of methionine[5,6]. An earlier preliminary account[7] suggested that glutamate was the source of the carbon atoms of the pyrroline moiety. On the basis of these observations it has been proposed that the parent ring system (**3**) is formed from the interaction of an acetate unit with a γ-activated form of glutamate and that this intermediate acts as the precursor of all the carbapenems[5]. The sequence of events leading to the introduction of the substituents at C_2 and C_6 remains somewhat obscure, although the Michael-type addition of thiol derivatives at C_2 and subsequent oxidation have been implicated[4,5,8~10]. The compound, sodium carbapen-2-em-3-carboxylate (**3a**) has been isolated from species of the bacteria *Erwinia* and *Serratia*, as its *p*-nitrobenzyl ester (**3b**)[11], and recently shown to possess the same absolute configuration at C-5 as the products from *Streptomyces*[12].

In a recent communication[13] we described the isolation and characterisation of the novel carbapenam compounds **4** and **5** as their *p*-nitrobenzyl esters, which were detected as a consequence of a study on the role of L-glutamate in the biosynthesis of the parent carbapenem **3a**. This paper describes the details of the incorporation of radiolabelled glutamate into the β-lactams **3**, **4** and **5**, as well as the incorporation of [1,2-$^{13}C_2$]acetate into **3** and **4**.



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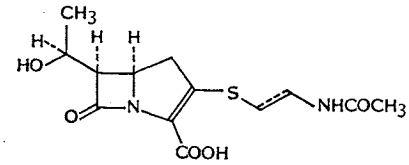

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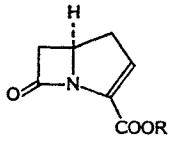
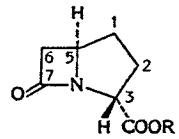
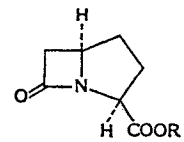


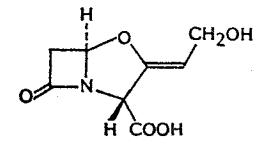
3a R = Na
3b R = PNB



4a R = PNB
4b R = Na



5a R = PNB
5b R = Na



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PNB=*p*-Nitrobenzyl.

Further to our interest in the biosynthesis of the olivanic acids[8] and other β-lactams[14], we considered it important to establish that the parent system was indeed glutamate derived. The production of **3a** by *Serratia* sp. ATCC 39006 was demonstrated by means of a β-lactamase induction assay procedure[15] and monitored by HPLC using an authentic sample of synthetic racemic material[16] as a standard. The susceptibility of **3a** to the β-lactamase II from *Bacillus cereus* provided a valuable additional identification procedure.

Preliminary experiments established that in a defined medium the antibiotic **3a** was produced in parallel with growth (Fig. 1). L-[*U*-^{14}C]Glutamate was fed to growing cells of the organism and after 24 hours the culture supernatant was ion-pair extracted (*via* Aliquat 336[17] in CH_2Cl_2) with back extraction into sodium iodide solution. The back extract was examined by HPLC monitored at 260 nm and fractions (taken every 15-second intervals) assayed for radioactivity to measure uptake of label into **3a**. Fig. 2a shows the plot of radioactivity against the fractions and the UV absorption trace; Fig. 2b shows the same plot but after treatment of the back extract with β-lactamase II. From these results it was apparent that glutamate was incorporated into **3a**.

In a separate experiment L-[*U*-^{14}C]glutamate (specific activity 50,715 dpm/μmol) was added at the time of inoculation to *Serratia* sp. ATCC 39006 in the defined medium. After 24 hours ion-pair extraction of the culture filtrate, followed by HPLC afforded the carbapenem (**3a**, 41,283 dpm/μmol). Since the defined medium contains no source of glutamate other than that added the molar specific activity 0.81 of **3a** suggested that the label was essentially incorporated intact. It was evident from Fig. 2 that a compound labelled by glutamate and having a similar retention time to **3a** was present. This component lacked absorption at the monitored wavelength, was stable to β-lactamase and was subsequently shown to be inactive in antibacterial assays.

Ion-pair extraction of culture filtrate, followed by derivatisation with *p*-nitrobenzyl bromide and careful chromatography afforded the crystalline *p*-nitrobenzyl ester (**3b**), together with two new isomeric esters **4a** and **5a**. **4a** and **5a** possessed molecular formulae two mass units greater than **3b** and were present in the relative ratio of approximately 9 : 1. The IR spectra of **4a** and **5a** showed strong

Fig. 1. Time course of cell growth and production of carbapenem **3a** by *Serratia* sp. ATCC 39006.
● Absorbance, □ antibiotic.

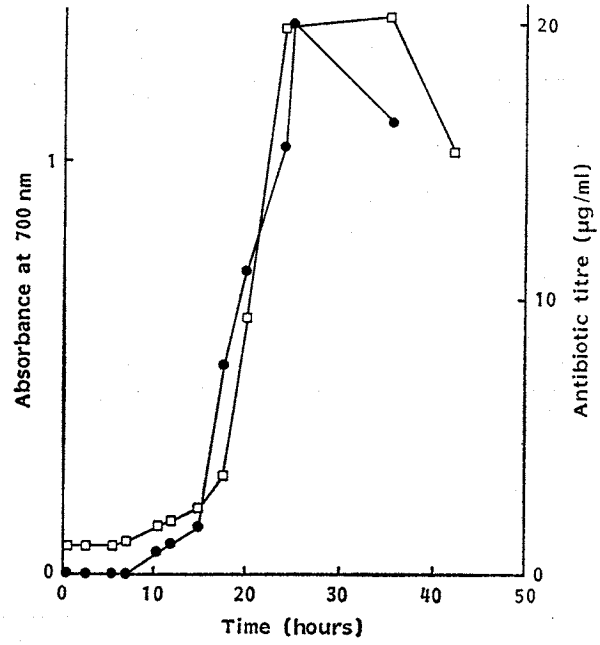
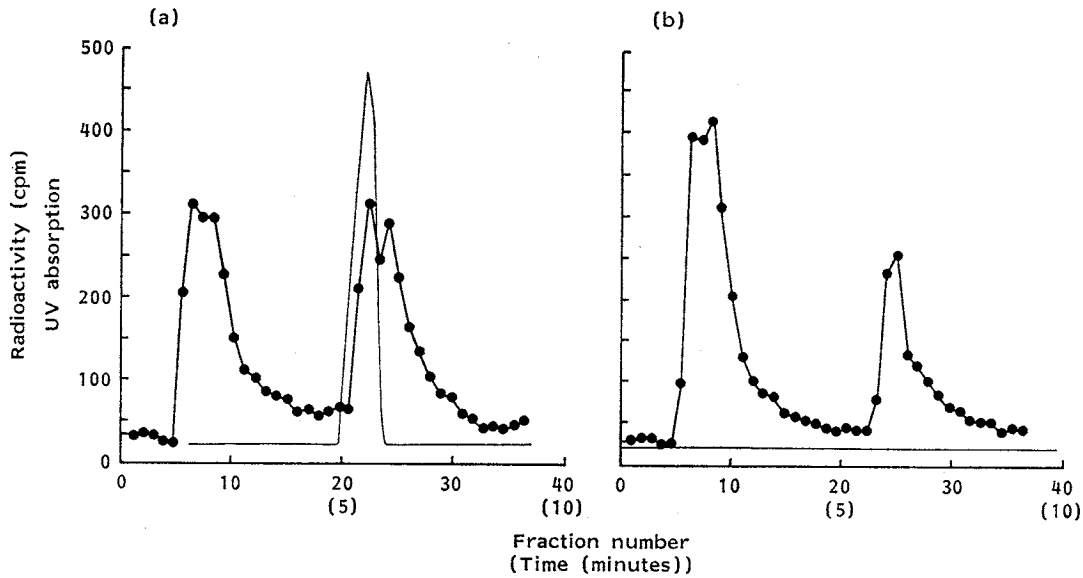


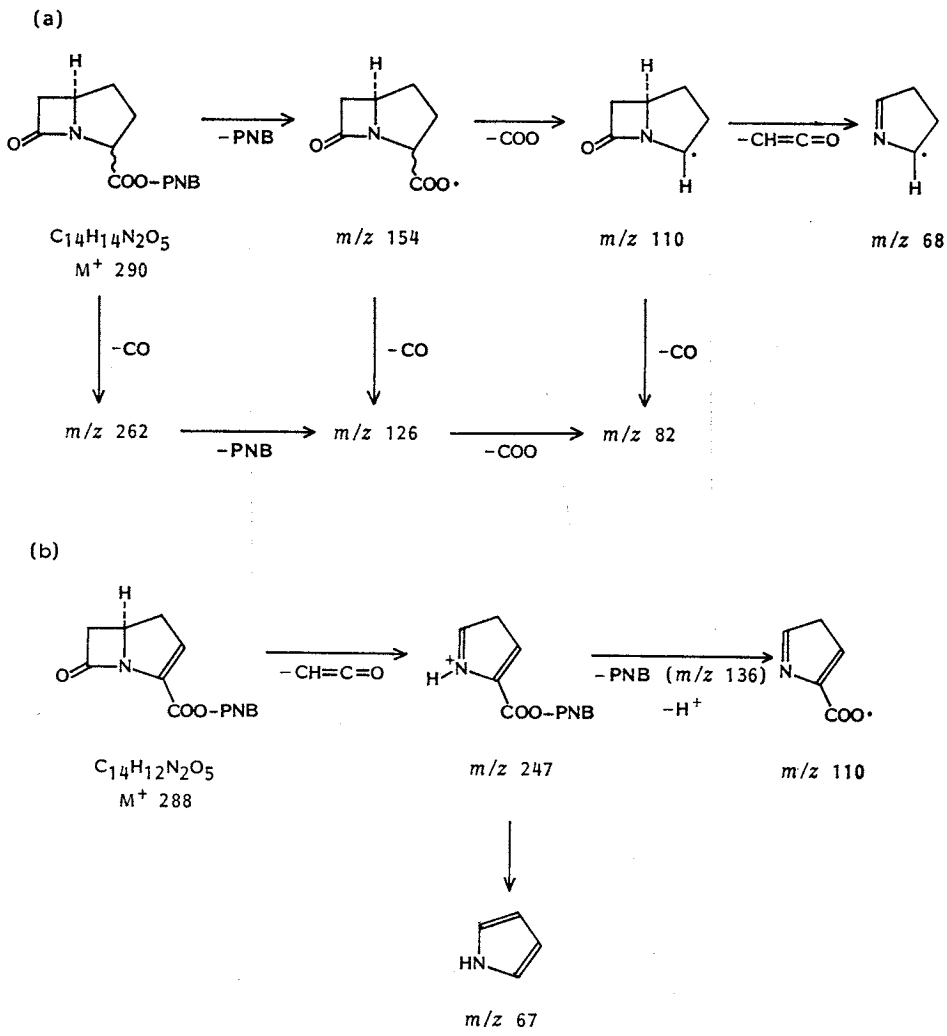
Fig. 2.
(a) Products from ion-pair extraction of the *Serratia* sp. ATCC 39006 culture filtrate.
(b) The same products after treatment with *Bacillus cereus* β-lactamase II.



— Absorption at 260 nm (arbitrary units), ● plot of radioactivity against fractions taken every 15 seconds.

HPLC was carried out using a C_{18} reverse phase column eluting with 0.1 M NaH_2PO_4 buffer pH 4 at 2 ml/minute and showed a single peak for **3a** with a retention time of 310 seconds.

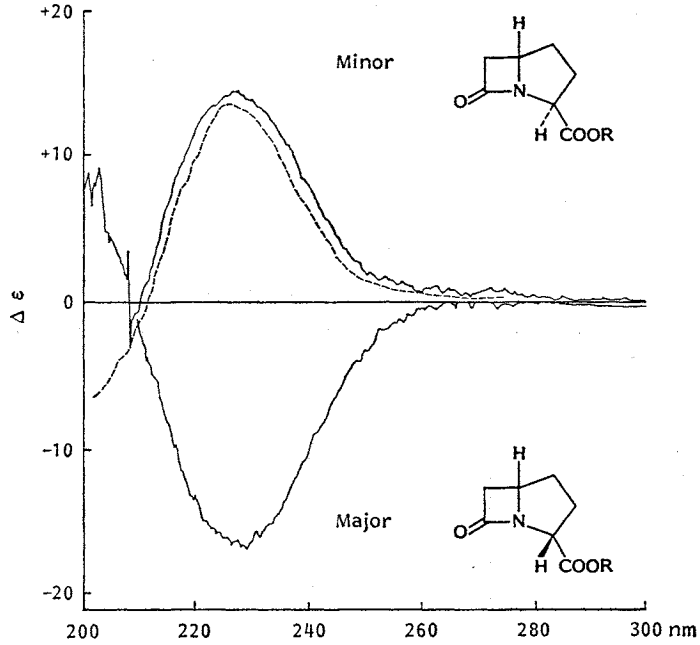
Scheme 1. MS fragmentation for the carbapenams **4a** and **5a** (a), and **3b** (b).



carbonyl stretching frequencies centred at 1760 and 1745 cm^{-1} respectively. A comparison of the electron impact mass spectral (EI-MS) fragmentation patterns with that of **3b** (Scheme 1) showed features indicative of β-lactams. These data together with the ^1H NMR spectra and a chemical correlation with **3b**[13], identified the major isomer as the carbapenam **4a** and the minor isomer as the C_3 epimer **5a**.

Catalytic hydrogenation of the natural carbapenem **3b** of known absolute configuration using Pd/C under controlled conditions afforded **5a** and **4a** in the ratio of approximately 9:1 (the reverse of that obtained from the natural source). The CD spectra of the two samples of **5a** *i.e.* derived from the natural source and by hydrogenation of **3a** showed identical positive Cotton effects at 230 nm, establishing the full absolute configuration for **5a** (Fig. 3). Insufficient **4a** was isolated from the hydrogenation experiment for direct chiral comparison with the natural material. However the CD spectrum of natural **4a** displayed a negative Cotton effect at 230 nm and a mirror image relationship with that of **5a**, strongly supporting the shown epimeric configuration at C_3 (Fig. 3). These results

Fig. 3. CD data of carbapenams.



—— Major and minor isomers (natural source), ------ major isomer from hydrogenation of carbapenem.

Table 1.

(a) ^{13}C chemical shifts of **3b**, **4a** and **5a** in ppm.

Carbon No.	3b	4a	5a
1	36.50	31.19	29.98
2	133.12	35.41	36.59
3	134.99	59.00	59.63
5	51.34	53.19	53.37
6	45.71	42.70	41.70
7	176.71	176.14	173.32
8	160.01	170.95	169.97
9	65.43	65.43	65.93
10	142.66	142.50	142.54
11, 15	128.22	128.39	128.90
12, 14	123.60	123.87	123.90
13	148.60	148.54	148.71

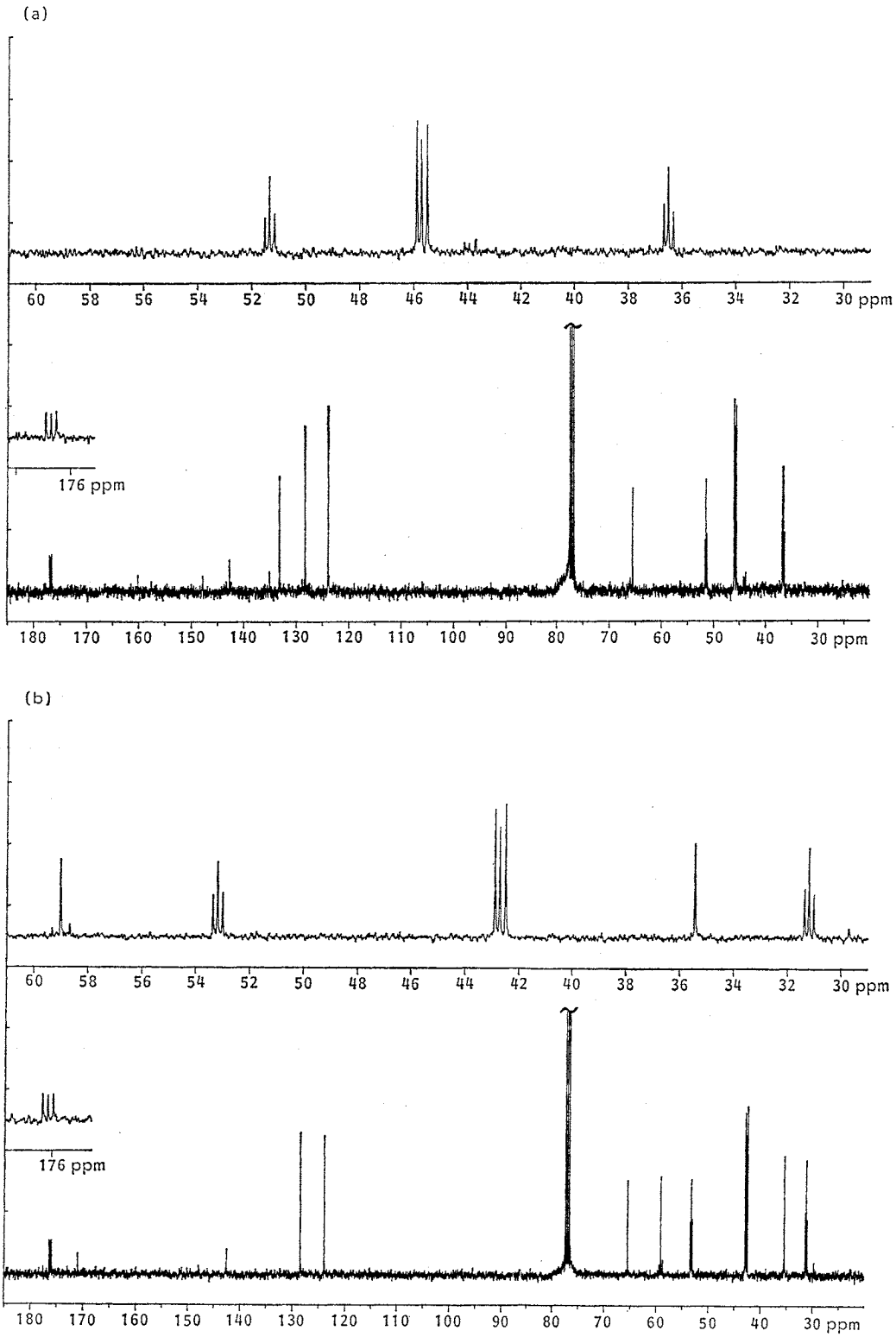
(b) ^{1}H chemical shifts of **3b**, **4a** and **5a** in ppm.

Carbon No.	3b	4a	5a
1	2.97,	2.31,	2.16,
	2.79	1.56	1.81
2	6.60	2.60,	2.38
		2.28	
3	—	4.48	3.97
5	4.31	3.88	3.73
6	3.53,	3.31,	3.14,
	3.01	2.67	2.76
9	5.43,	5.52	5.32,
	5.28		5.25
11, 15	7.61	7.52	7.56
12, 14	8.26	8.24	8.24

established that *Serratia* produces two novel carbapenams **4b** and **5b** both lacking antibacterial activity and resistant to β-lactamase, which are the epimeric dihydro derivatives of the parent carbapenem **3a**. It is noteworthy that **4b** possesses the same absolute and relative configurations at the C-3 and C-5 positions as the natural penicillins, whereas the minor isomer is epimeric at C-3 and has the unusual L-configuration.

The incorporation of L-[*U*-^{14}C]glutamate into all three compounds suggested that at least part of the molecules was glutamate derived. To provide more definitive data to confirm this conclusion and also identify the origin of the β-lactam carbons the incorporation of [1,2-$^{13}C_2$]acetate into all three

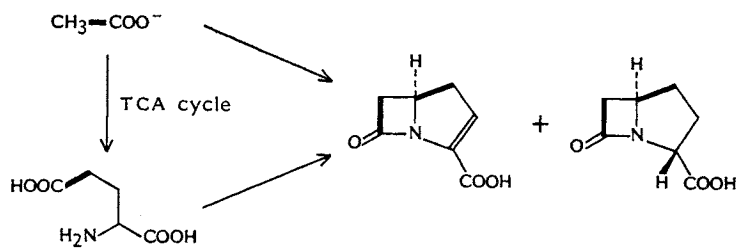
Fig. 4. ^{13}C NMR spectra of **3b** (a) and major isomer **4a** (b) after incorporation of [1,2-$^{13}C_2$]acetate.



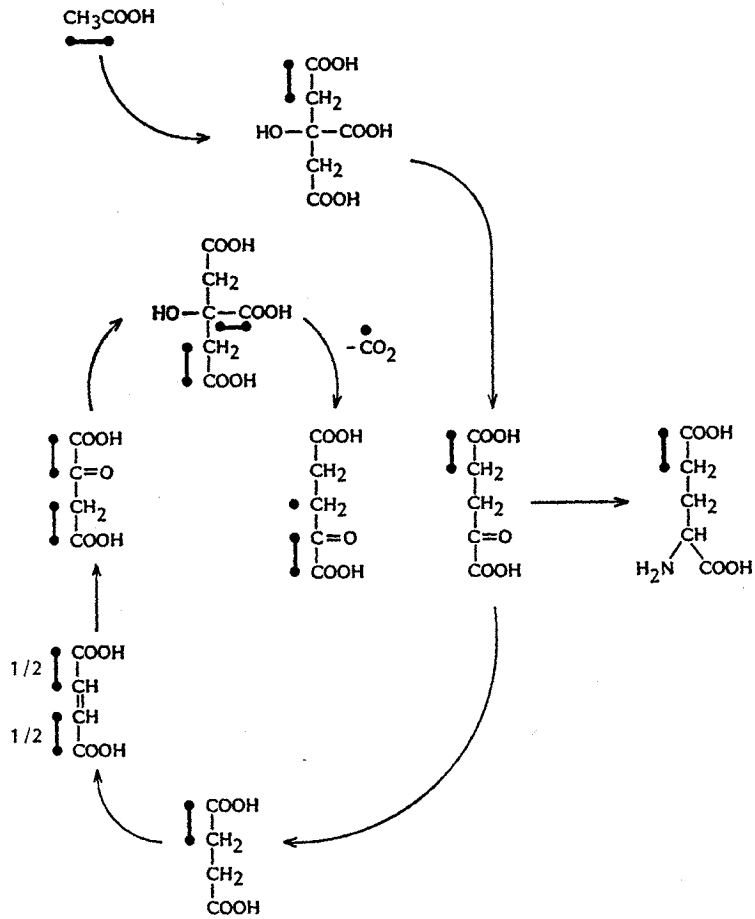
compounds was investigated. A series of preliminary experiments, whereby [1-^{14}C]acetate and defined concentrations of cold acetate were fed to growing cells and the specific activity of **3b**, **4a** and **5a** subsequently determined, allowed the optimal concentration of acetate for maximum incorporation to be established. For the feeding experiment up to 50 mg of [1,2-$^{13}C_2$]acetate was added in intervals to each of 200 ml×10 fermentation batches. Isolation of the *p*-nitrobenzyl ester (**3b**) and the major dihydro isomer (**4a**) was as previously described. The ^{13}C NMR spectra of these isotopically enriched compounds are shown in Fig. 4 and the assignments of the natural abundance spectra of **3b**, **4a** and **5a** are given in Table 1. The spectrum of the carbapenem derivative **3b** in Fig. 4a shows characteristic doublet signals at the C-6 and C-7 positions (centred at 176.71 and 45.71 ppm, J=38.2 Hz) clearly identifying a substantial incorporation of an intact acetate unit into the β-lactam ring. In addition a significant but lesser incorporation of an acetate unit at C-1 and C-5 (doublets centred at 51.34 and 36.50 ppm, J=35.0 Hz) is apparent. Similarly from the spectrum of the carbapenam ester (**4a**) shown in Fig. 4b, pairs of doublets centred at 176.14 and 42.70 ppm, J=38.1 Hz and 53.19 and 31.19 ppm J=34.5 Hz demonstrate the same level of incorporation of intact acetate at C-6/C-7 and C-1/C-5 respectively. This information provides evidence that the biosynthesis of **3a** and **4b** are intimately related and derived from common precursors. It would appear that C-6/C-7 in both molecules are derived from an intact acetate unit (Scheme 2) as has been observed in the case of thienamycin (**1**)[5]. An incorporation of an acetate unit at C-1/C-5 is also consistent with the earlier observation that glutamate is the precursor of these systems. A single turn of the tricarboxylic acid (TCA) cycle with doubly labelled acetate leads ultimately to [4,5-$^{13}C_2$]glutamate whereby C-5 of glutamate is derived from C-1 of acetate as illustrated in the Scheme 3. This would be consistent with the pyrroline and pyrrolidine rings of **3a** and **4b** respectively being derived directly from glutamate. Further evidence supporting this conclusion stems from a more detailed examination of the spectrum shown in Fig. 4b. The signal for C-3 displays a small doublet centred around 59.00 ppm (J=66.4 Hz). The corresponding coupled doublet is not observed above the noise but can be assigned to the low intensity quaternary C-8 signal since a similar coupling constant has been reported between C-3 and C-10 in clavulanic acid (**6**)[14]. This can be interpretated by labelled α-ketoglutaric acid undergoing a second turn of the TCA cycle (see Scheme 3).

These results coupled with the earlier findings on the biosynthesis of thienamycin can be interpretated in terms of a number of possible interrelated pathways. The original proposal (Scheme 4, pathway (a)) involved the condensation of acetyl-*S*-CoA with γ-glutamylphosphate to afford an hypothetical monocyclic intermediate **7**, tautomerisation to **8**, followed by ring closure leading to the carbapenem **3**[5]. While our findings would accord with this proposed pathway for the formation of **3**, the detection of the carbapenams **4b** and **5b** in both *Serratia* and *Erwinia* species which also produce the car-

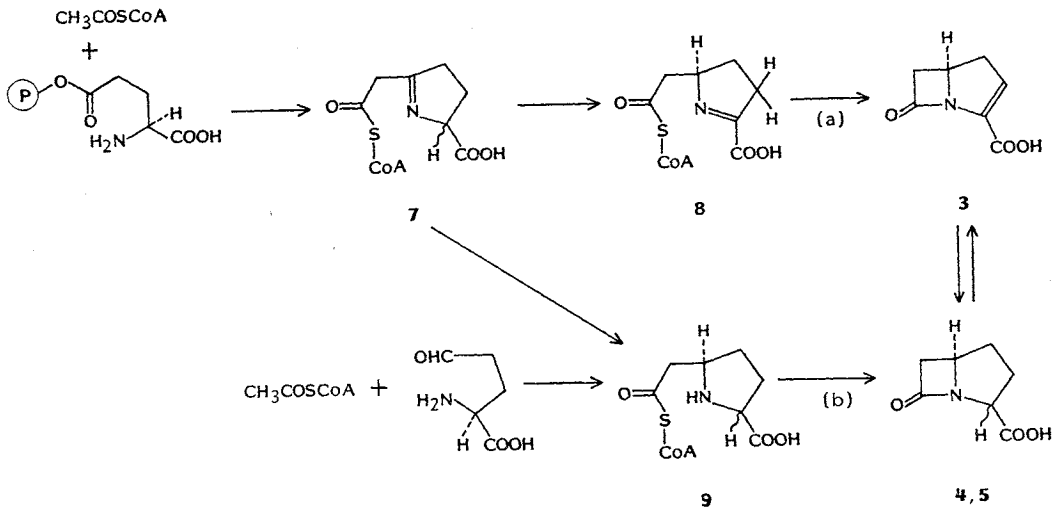
Scheme 2. Incorporation of [1,2-^{13}C]acetate into **3a** and **4b**.



Scheme 3. Incorporation of [1,2-$^{13}C_2$]acetate into glutamate *via* the TCA cycle.



Scheme 4. Possible biosynthetic pathways to carbapenems and carbapenams.



bapenem **3** raises other interesting possibilities as illustrated in Scheme 4. There is the obvious inference that the carbapenams could be the end-products formed by the reduction of **3**. An alternative possibility is that either one or both of the dihydro compounds **4** and **5** are in fact the precursors of the carbapenem **3**. This would imply that they result from a putative intermediate **9** which itself could be derived either by reduction of **7** or by the direct condensation of acetyl-*S*-CoA and glutamate semi-aldehyde (pathways (b)). The experimental data described in this paper and in the literature[5] cannot distinguish between pathways (a) or (b). Nor is it possible to rule out that the both pathways function independently. It is intriguing to note that the conversion of both the hypothetical intermediates **8** and **9** to the carbapenem and carbapenams respectively can be regarded in essence as reverse β-lactamase reactions.

Experiments are currently in hand to discover which of the proposed pathways operate in *Serratia* and to explore some of the implications.

Experimental

^{14}C-Labelled acetate and glutamate, and [1,2-$^{13}C_2$]acetate (92 atom % ^{13}C) were obtained from Amersham International pLc, UK. The specific activity of ^{14}C-labelled compounds was estimated in scintillation fluid (Optiphase 'Safe', Fisons, Loughborough, UK) using a LKB-Wallac 1219 Spectral Liquid Scintillation Counter (Wallac, Oy, Turku, Finland). IR spectra were determined using a Perkin-Elmer 983 spectrometer in potassium bromide micro-discs. GC-Mass spectroscopy was performed using a PYE 104 gas chromatograph coupled to a VG 7070F mass spectrometer operated in the EI mode at low resolution (1,000 R.P.). A 25 metre BP 1 (OV 1 equivalent) fused silica capillary column was used for the analysis.

Natural abundance NMR spectra were run at 297 K on a Bruker AM 400 operating at a ^{13}C resonance frequency of 100.614 MHz. The spectra were acquired in 5 mm tubes in a ^{13}C/^{2}H-dual probe, using a sweep width of 27,777.778 Hz over 64 K data points, with $CDCl_3$ as solvent and TMS as internal reference. A 1.75-Hz line broadening was applied prior to Fourier transformation. Assignment was aided by spin-echo and DEPT spectra, both DEPT-90° and -135° variants, obtained by using standard Bruker software.

The spectra of the isotopically enriched compounds were obtained using broad band decoupling, and a relaxation delay of 2.5 seconds, since the multipulse acquisitions are not tuned to correctly refocus 1 bond carbon-carbon couplings.

The proton assignments were extracted from spectra acquired on the same instrument using a sweep width of 5,208 Hz over 32 K data points at 297 K in $CDCl_3$ - TMS.

Assay Procedures

(a) HPLC: The system comprised a Waters Associates Ltd. (Northwich, UK) Model 6000A solvent delivering system and a Rheodyne (Cotati, California, U.S.A.) injector connected to a Hichrom 4.9×250 mm Spherisorb S50082 reverse phase column (Hichrom Ltd., Reading, UK), and monitored with a Cecil Model 212 UV spectrophotometer (Cecil Instruments, Cambridge, UK) *via* an 8-μl flowcell with a 10-mm path length at 260 nm. The eluant was 100 mM KH_2PO_4 - NaH_2PO_4 buffer, pH 7.0 at a flow rate of 1 ml/minute; **3a** had a retention time of 6.5 minutes. An authentic sample of synthetic racemic **3a**, kindly supplied by BATESON *et al.*[16], was used as standard.

Identification of **3a** was facilitated by treatment of assay samples with the *B. cereus* β-lactamase II enzyme which degraded **3a**, prior to HPLC analysis.

p-Nitrobenzyl esters **3b**, **4a** and **5a** were assayed in a similar manner except that the eluant was 40% CH_3CN in water and the absorbance was measured at 270 nm; the retention times for **3b**, **4a** and **5a** were 15, 12.5 and 13.5 minutes respectively, at a flow rate of 1 ml/minute.

(b) TLC-β-lactamase Induction: The presence of **3a** was also monitored using the β-lactamase induction assay of SYKES and WELLS[15]. Samples were assayed directly by the agar diffusion method

or following TLC. The TLC plates (Kieselgel $60F_{154}$, Merck, Dermstadt, West Germany) were run in hexane - acetone (2 : 1).

Fermentation

Serratia sp. ATCC 39006 was maintained on Nutrient Agar (London Analytical and Bacteriological Media Ltd., Salford, UK) and stored at 4°C. A seed culture was obtained by inoculating a loop full of surface growth from an agar slant into 250-ml Erlenmeyer flasks containing 50 ml of seed medium. The seed medium contained Neutralised Soya Peptone (2%) (Oxoid Ltd., London, UK) and sucrose (0.2%). Flasks were incubated at 26°C on a rotatory shaker at 240 rpm for 24 hours. The resulting seed culture was used as inoculum (2%) for the fermentation stages. Fermentations were carried out as appropriate in 50 ml, 1 or 2-litre Erlenmeyer flasks containing 10, 100 and 200 ml medium respectively. The fermentation medium contained L-glutamine 0.5%, NH_4Cl 0.075%, K_2HPO_4 0.2%, NaCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.0025%, $CaCO_3$ 0.025%, $CoCl_2 \cdot 6H_2O$ 0.001% and sucrose 1%; flasks were incubated at 26°C on a rotary shaker.

Preparation of Carbapenem **3b** and Carbapenam **4a** and **5a**

Whole fermentation broth was clarified by centrifugation to yield culture supernatant fluid (2 litres) which was shaken with 100 mM Aliquat 336 (Aldrich Chemical Co., Ltd., Gillingham, UK) in dichloromethane (400 ml). The organic phase was separated, dried over $MgSO_4$ and treated with *p*-nitrobenzylbromide (4 g) and the solution stirred at room temperature for 2 hours. Application of the TLC/β-lactamase induction assay to the solution indicated the presence of ester (**3b**). Concentration of the solution gave an oil, which after solution in 20% EtOAc in *n*-hexane, was chromatographed on a silica (Silica Woelm TSC, Woelm Pharam GmbH, and Co., Eschwege, West Germany) column, eluted with 50% EtOAc - *n*-hexane. Fractions (20-ml) were collected and assayed by TLC; **3b**, **4a** and **5b** cochromatographed and were detected as a single unresolved spot (Rf 0.35) by UV. The fractions containing **3b**, **4a** and **5a** were pooled, evaporated to yield an oil which was dissolved in 50% CH_3CN in water and applied (4×500 μl injections) to a semi-preparative version of the HPLC analytical column.

Fractions corresponding to **3b**, **4a** and **5a** were pooled separately and freeze-dried to give 2.5 mg of *p*-nitrobenzyl (5*R*)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (**3b**) as a colourless crystalline solid: IR (KBr) 1778 (β-lactam CO), 1728 (ester CO), 1658, 1607, 1522 cm^{-1}; 2.5 mg of *p*-nitrobenzyl (3*R*,5*R*)-7-oxo-1-azabicyclo[3.2.0]heptan-2-carboxylate (**4a**) as a white solid: IR (KBr) 1760 (β-lactam CO), 1749 (ester CO) cm^{-1}; 0.3 mg of *p*-nitrobenzyl (3*S*,5*R*)-7-oxo-1-azabicyclo[3.2.0]heptan-2-carboxylate (**5a**) as a pale cream gum: IR (KBr) 1745 broad (β-lactam CO and ester CO) ^1H and ^{13}C NMR spectra are presented in Table 1.

Incorporation of L-[*U*-^{14}C]Glutamate into Carbapenem **3a**

L-[*U*-^{14}C]Glutamate was added to a 50-ml flask containing the defined medium (10 ml) inoculated with *Serratia* sp. ATCC 39006. After incubation at 26°C for 24 hours the culture was centrifuged and the supernatant extracted with 100 mM Aliquat 336 solution in CH_2Cl_2 (5 ml). The CH_2Cl_2 layer was separated, extracted with 200 mM NaI solution (1 ml), and this aqueous back extract was submitted to HPLC assay at 260 nm and fractions (collected at 15-second intervals) were also assayed for radioactivity. The plot of radioactivity against fraction number and UV absorbance trace is given in Fig. 2a. Treatment of the back extract with β-lactamase II, derived from *B. cereus* and re-determining the UV absorbance and radioactivity of the fractions resulted in the plot shown in Fig. 2b.

Effect of [1-^{14}C]Acetate Concentration on Its Incorporation into **3b**, **4a** and **5a**

To 3×200 ml fermentations 10, 40 and 100 mg of [1-^{14}C]acetate (137,500 dpm/μmol) were added at periods during the logarithmic growth phase, *i.e.* 25% at inoculation 10% at A_{700} 0.3,

Table 2. Effect of [1-^{14}C]acetate concentration on its incorporation into esters **3b**, **4a** and **5a**.

Concentration of [1-^{14}C]acetate (mg/200 ml)	Specific activity (dpm/μmol)		
	3b	**4a**	**5a**
10	3,108	1,952	2,760
40	6,150	10,600	7,083
100	23,516	22,218	19,892

20% at A_{700} 0.5 and 45% at A_{700} 0.7. The fermentation was stopped at the end of the logarithmic phase and esters **3b**, **4a** and **5a** were isolated *via* ion-pair extraction as described earlier. Yields of the respective esters were determined by UV spectroscopy and the specific activity estimated. The extent of incorporation of acetate with respect to the concentration of acetate fed is given in Table 2.

Incorporation of [1,2-$^{13}C_2$]Acetate into **3b** and **4a**

[1,2-$^{13}C_2$]Acetate (50 mg) with [1-^{14}C]acetate (20 μCi) as tracer, was added in portions to a flask containing 200 ml growing culture; ten such flasks provided 2 litres of broth which were extracted in the manner outlined above to provide esters **3b** and **4a**. The ^{13}C NMR spectra of these compounds showing the various enrichments are given Fig. 4.

Hydrogenation of **3b**

0.8 mg of **3b** dissolved in 2 ml of ethanol in a 100-ml round-bottomed flask was stirred whilst being connected to a Peteric Gas Control Apparatus Type 2020 (Hydrogenator), Peteric Instrumentation Ltd. 0.4 mg of Pd/C was added (Palladium, 10% on carbon, Lancaster Synthesis Ltd., Morecombe, UK) and hydrogenation was followed by sampling at intervals through a rubber septum and analysing by TLC on silica with 50% hexane in 2-propanol. The production of **5a** stopped after about 1 hour and another 0.4 mg Pd/C was added. Again, no more **5a** was produced after 1 hour and a further 0.4 mg Pd/C added. Hydrogenation was stopped when virtually all the **3b** had disappeared and the Pd/C removed by filtration through Celite 545 (Fluka AG, Switzerland), washing with ethanol. The ethanol was removed by rotary evaporation to dryness under vacuum at 30°C and the sample redissolved in 40% CH_3CN in water. HPLC analysis showed that hydrogenation of **3b** had produced **5a** and **4a** in a ratio of approximately 9 : 1 (the reverse of that obtained from the natural source). **5a** was purified by HPLC and freeze-dried, yielding approximately 0.25 mg.

Addendum in Proof

It was provisionally proposed that **4a** was epimeric to **5a** at C_3[13]. A recent unambiguous synthesis (BYCROFT, B. W. and R. CHHABRA; unpublished results) has established that the absolute configuration of **4a** is (3*S*,5*S*).

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