STUDIES ON THE BIOSYNTHESIS OF CLAVULANIC ACID

II. CHEMICAL DEGRADATIONS OF ^{14}C-LABELLED CLAVULANIC ACID

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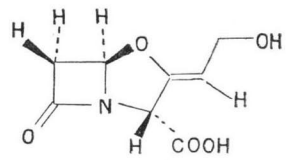
(Received for publication August 9, 1979)

Two chemical degradations of clavulanic acid are described which are useful for locating label in ^{14}C-clavulanate. In the first, the β-hydroxyethylidene side chain of *p*-bromobenzyl clavulanate is removed by ozonolysis to give *p*-bromobenzyl (2R, 5R)-3,7-dioxo-4-oxa-1-azabicyclo [3.2.0] heptane-2-carboxylate. The second involves the reaction of *p*-bromobenzyl clavulanate with dibenzylamine in methanol, to isolate the three β-lactam carbons as methyl *trans*-3-(*N,N*-dibenzyl)amino acrylate. These techniques were used to degrade clavulanic acid derived from fermentations fed with 2-^{14}C-acetate or universally ^{14}C-labelled glycerol. The amount of label retained in the degradation products was in agreement with the distribution of ^{13}C in clavulanic acid derived from 2-^{13}C-acetate, or 1,3-$^{13}C_2$-glycerol, as observed by ^{13}C-NMR.

When studying the biosynthesis of a natural product, it is normal practice to determine the % incorporation of ^{14}C-labelled compounds which are possible precursors of the product under investigation. However, unless an unusually high incorporation is observed, this type of experiment often yields results of little value, as many compounds can be extensively metabolised before being incorporated into the product. This was found to be the case with the initial studies on the biosynthesis of clavulanic acid. When ^{14}C-labelled precursors were fed to *Streptomyces clavuligerus* fermentations, most were found to be incorporated into clavulanic acid to some extent. However, it was not possible to draw any meaningful conclusions from these experiments, and it became apparent that this type of experiment would only be useful if the position of ^{14}C-label could be located in the product.

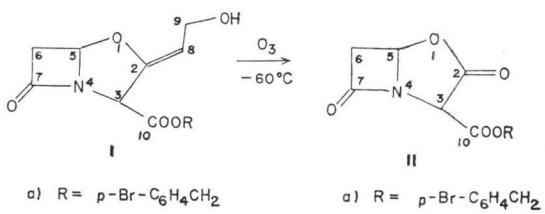
Fig. 1. Clavulanic acid



The following two chemical degradations of clavulanic acid were found useful for this purpose.

Degradation by Ozonolysis

Scheme 1.



Carboxylic esters of clavulanic acid can be degraded by ozonolysis to give the bicyclic lactone (**II**)[1]. For radio labelled studies, the *p*-bromobenzyl ester (**Ia**) was preferred as this is a crystalline solid, and could, therefore, be recrystallised to constant radioactivity. The product (**IIa**) is also crystalline.

2-^{14}C-Acetate was fed to a *S. clavuligerus* fermentation and the resulting clavulanic acid isolated

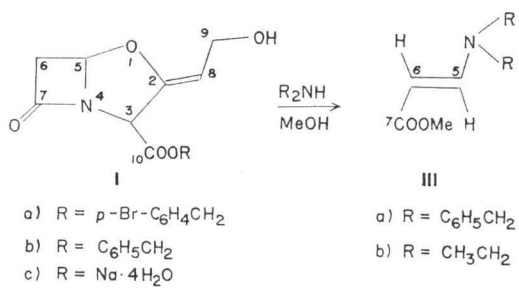
Table 1. Incorporation and distribution of ^{14}C-label in clavulanic acid.

Precursor	Amount added to fermentation μCi	Isolated as	Specific activity of product μCi/mmol	Incorporation of label %	Degraded with	Specific activity of		% Retention of label in degradation product
						Starting material (Ia) μCi/mmol	Degradation product (IIa) or (IIIa) μCi/mmol	
2-^{14}C-Acetate	500	Ia	4.64	2.6	Ozone	2.88*	2.58	89.6
	50,000	Ic	1,458	4.2	Dibenzylamine	44.05*	17.06	38.7
	26,000	Ic	362	6.3	Dibenzylamine	91.52*	35.29	38.6
Universal ^{14}C-glycerol	250	Ia	13.2	9.0	Dibenzylamine	5.16*	4.81	93.2
	9,700	Ic	194	3.8	Dibenzylamine	89.70*	85.50	95.3

* After dilution with unlabelled (Ia)

as **Ia**. When this was degraded with ozone it was found that 89.6% of the radioactivity in **Ia** was retained in the degradation product (Table 1). This was in agreement with previous ^{13}C-NMR studies on ^{13}C-benzyl clavulanate derived from 2-^{13}C-acetate, where it was observed that C-2, C-3, C-5, C-6, C-7, C-8 and C-10 were enriched, whereas there was little enrichment of C-9[2].

Scheme 2.



Degradation with Dibenzylamine

Esters of clavulanic acid also undergo reaction with secondary amines in methanol to yield the corresponding *trans*-aminoacrylates (DAVIES, J. S.; J. GOODACRE & T. T. HOWARTH: to be published). For this degradation, clavulanic acid was again isolated as the crystalline *p*-bromobenzyl ester (**Ia**). Dibenzylamine was the preferred degrading agent as this gives a crystalline product (**IIIa**)[3].

To test this method of degradation, four *S. clavuligerus* fermentations were performed, two fed with 2-^{14}C-acetate and two with universally ^{14}C-labelled glycerol. The clavulanate was isolated as **Ia** in each case, and degraded with dibenzylamine to give **IIIa**. The results are shown in Table 1.

The retentions of radioactivity in **IIIa** for the two precursors were in agreement with the distribution of label observed in clavulanic acid derived from ^{13}C-labelled precursors, where 1,3-$^{13}C_2$-glycerol was found to be incorporated almost exclusively in the β-lactam ring, whereas label from 2-^{13}C-acetate was incorporated into 7 out of 8 of the clavulanate carbons[2].

Further evidence that this type of degradation isolates the β-lactam carbons was provided when benzyl clavulanate (**Ib**) derived from 1,3-$^{13}C_2$-glycerol was degraded with diethylamine in methanol to give **IIIb**. When **Ib** and **IIIb** were examined by CMR, the ^{13}C-^{13}C spin-spin coupling observed between carbons 5 and 7 in **Ib**[2] was found to be retained in **IIIb**. (J. S. DAVIES, Personal communication). It was of interest that although the extent of incorporation varied between experiments (*e.g.* 3.8% to 9.0% for universal ^{14}C-glycerol), the distribution of label in the product remained constant.

Comment

Previously reported studies[2] showed the close relationship of clavulanic acid biosynthesis with primary metabolism and indicated that feeding carbon-labelled compounds which could be metabolised *via* the TCA cycle would result in labelling both the β-lactam ring and oxazolidine ring carbon skeletons. The two degradations described above, therefore, provide a useful method for determining whether ^{14}C-labelled precursors have been specifically or generally incorporated into clavulanic acid.

Experimental

General

Melting points were recorded on a Kofler hot stage apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 157 spectrophotometer. ^1H NMR spectra were measured at 60 MHz on a Perkin-Elmer R12A spectrometer, or at 90 MHz on a Perkin-Elmer R32 spectrometer, using tetramethylsilane as internal reference. Mass spectra were obtained on an A.E.I. MS 9 spectrometer operating at 70 eV. The radioactivities of *p*-bromobenzyl clavulanate and its degradation products were determined by accurately weighing duplicate or triplicate samples of the solids in aluminium boats and adding these directly to vials of liquid scintillant consisting of 0.8% Omnifluor® (New England Nuclear, Boston, USA), 30% ethoxyethanol, 70% toluene. The vials were counted on a Packard Tricarb 3380 liquid scintillation spectrometer. Ozonisations were carried out using a Nu Aire ozone generator. Light petroleum refers to the fraction of b.p. 60~80°C. Organic solutions were dried over magnesium sulphate where necessary. Analytical TLC was carried out on precoated glass plates (Merck; silica gel 60 F_{254}) using ethyl acetate - cyclohexane 1:1 as eluant. Detecting spray reagents used for TLC were dilute aqueous potassium permanganate ($KMnO_4$) and 2% triphenyltetrazolium chloride in 1.0 M sodium hydroxide in 50% aqueous methanol (TTC).

Radiochemicals

Universally ^{14}C-labelled glycerol (32 mCi/mmol) and 2-^{14}C-sodium acetate (58.3 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Preparation of unlabelled reference materials

(1) *p*-Bromobenzyl clavulanate (**Ia**)

A solution of sodium clavulanate tetrahydrate (2.9 g; 10 mmol) and α,*p*-dibromotoluene (2.5 g; 10 mmol) in dimethylformamide (50 ml) was stirred at ambient temperature for 5 hours. The solvent was removed *in vacuo* and the residue partitioned between ethyl acetate and water. The aqueous phase was re-extracted with ethyl acetate and the combined organic extracts were washed with brine, dried and evaporated to a solid, which was recrystallised from methylene chloride - light petroleum to give **Ia** (2.2 g, 60% yield) as colourless needles, Rf 0.16 ($KMnO_4$), m.p. 104°C, (lit[4] 103~104°C), $[\alpha]_D^{20}$ +57.8° (*c* 1.0, $CHCl_3$), (Found: C, 48.85; H, 3.93; N, 3.77; Br, 21.80%; M^+ 367.0052; $C_{15}H_{14}BrNO_5$ requires C, 48.93; H, 3.83; N, 3.80; Br, 21.70%; M 367.0055); ν_{max} (Nujol) 3250 (OH), 1795 (β-lactam C=O), 1738 (ester C=O), 1695 cm^{-1} (C=C); δ($CDCl_3$) 3.02 (1H, dd, J 17.5 and 1 Hz, 6-βCH), 3.45 (1H, dd, J 17.5 and 2.5 Hz, 6α-CH), 4.17 (2H, d, J 8Hz, 9-CH_2), 4.83 (1H, m 8-CH), 5.05 (1H, s, 3-CH), 5.11 (2H, s, CH_2Ar), 5.63 (1H, dd, J 2.5 and 1 Hz, 5-CH), 7.16, 7.47 (each 2H, d, J 9.5 Hz, Ar-H).

(2) *p*-Bromobenzyl 3,7-dioxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate (**IIa**)

A current of ozone was passed through a cold (−60°C) solution of *p*-bromobenzyl clavulanate (**Ia**) (1.5 g; 4 mmol) in ethyl acetate (100 ml), with stirring. After 1.5 hours the ozone was replaced by a stream of nitrogen and the temperature of the reaction allowed to rise slowly to ambient temperature. The solution was washed with water, brine, dried and evaporated *in vacuo* to yield a solid, which on recrystallisation from methylene chloride-light petroleum gave **IIa** (0.98 g; 71%) as colourless needles Rf 0.38 ($KMnO_4$), m.p. 105~106°C, $[\alpha]_D^{20}$ +141° (*c* 1.1, CH_3OH), (Found: C, 45.86; H, 3.06; N, 4.12; Br, 23.6%; M^+ 338.9743. $C_{13}H_{10}BrNO_5$ requires: C, 45.90; H, 2.96; N, 4.12; Br, 23.49%; M 338.9742); ν_{max} (Nujol) 1810~1790 (lactone C=O and β-lactam C=O), 1765 cm^{-1} (ester C=O); δ ($CDCl_3$) 3.23

(1H, dd, J 17.5 and 1Hz, 6 β-CH), 3.63 (1H, dd, J 17.5 and 3Hz, 6α-CH), 4.88 (1H, s, 3-CH), 5.16 (2H, s, CH_2Ar), 5.73 (1H, dd, J 3 and 1Hz, 5-CH), 7.18, 7.48 (each 2H, ABq, J 9Hz, Ar-H).

(**3**) Methyl *trans*-3-(*N*,*N*-dibenzyl)aminoacrylate (**IIIa**)

Methyl propiolate (0.42 g; 5 mmol) in dry methanol (10 ml) was stirred with dibenzylamine (0.98 g; 5 mmol) under nitrogen for 20 hours. The solvent was then removed *in vacuo* and the residual solid crystallised from toluene-petroleum ether, (1.2 g; 86%), Rf 0.49 ($KMnO_4$), m.p. 69°C (lit.[3] 68~69°C); ν_{max} (Nujol) 1678, 1604 cm^{-1}; $\delta(CDCl_3)$ 3.63 (3H, s, CO_2CH_3), 4.27 (4H, s, $N(CH_2C_6H_5)_2$), 4.77 (1H, d, J 13Hz, $CHCO_2CH_3$), 7.22 (10H, m, Ar-H), 7.77 (1H d, J 13Hz, $CHN(CH_2C_6H_5)_2$).

(**4**) Methyl *trans*-3-(*N*,*N*-dibenzyl)aminoacrylate (**IIIa**) from *p*-bromobenzyl clavulanate

Dibenzylamine (0.16 g; 0.8 mmol) was stirred with *p*-bromobenzyl clavulanate (0.1 g; 0.27 mmol) in dry methanol (10 ml) under an atmosphere of nitrogen for 19 hours. The solution was then evaporated to dryness *in vacuo*, the residue dissolved in a small quantity of ethyl acetate - cyclohexane 1: 1 and loaded onto a column of silica gel (Merck: silica gel 60; 70~230 mesh). The column was eluted with ethyl acetate - cyclohexane 1: 1. Fractions were examined by TLC. Fractions containing a component at Rf 0.49 ($KMnO_4$) were bulked, dried *in vacuo*, and recrystallised from toluene-light petroleum to give colourless needles (0.5 g, 66% yield), m.p. 68~69°C. This product was identical (IR and NMR) with the material prepared in (**3**) above.

Fermentations

The organisms used for clavulanic acid production were *Streptomyces clavuligerus* IT2[2], or *S. clavuligerus* SM 240, the latter being derived from *S. clavuligerus* ATCC 27064 by ultraviolet light mutation. Fermentations were carried out as published previously[2]. ^{14}C-Labelled precursors were added during the clavulanic acid production phase. Clavulanic acid accretion was then allowed to continue for a further 16~20 hours before the fermentations were harvested. Effluent air from each fermenter was passed through two Dreschel bottles each containing 300 ml ethanolamine to trap ^{14}C-carbon dioxide. Clavulanic acid titres were assayed by the automated assay described previously[2].

Isolation of ^{14}C-*p*-bromobenzyl clavulanate (**Ia**)

At harvest, the culture was centrifuged to remove the mycelium. The culture supernatant was freeze dried, and the resulting solid (typically 16 g) was reacted with excess α,*p*-dibromotoluene (5.0 g) in dimethylformamide (50 ml). After 3 hours, excess ethyl acetate (~500 ml) was added and insolubles filtered off. The ethyl acetate solution was reduced to an oil by rotary evaporation *in vacuo* and further purified by column chromatography using Sephadex LH20 (Pharmacea), (solvent system; cyclohexane - chloroform 1: 1) and/or by silica-gel column chromatography (Merck: silica gel 60, gradient elution with cyclohexane - ethyl acetate). Fractions containing pure *p*-bromobenzyl clavulanate, as judged by TLC (detecting spray reagents: TTC and $KMnO_4$), were bulked and dried *in vacuo*. The resulting solid was crystallised from methylene chloride-light petroleum. The ^{14}C-*p*-bromobenzyl clavulanate was then recrystallised to constant radioactivity. Purity was checked by TLC (as above) and the TLC plates were also scanned for radioactive impurities using a Pannax scanner (Pannax Equipment Ltd., Redhill, Surrey, U.K.).

All ^{14}C-*p*-bromobenzyl clavulanate samples produced *via* this route were identical with the unlabelled reference material as judged by TLC, melting point, mixed melting point and IR spectrum.

Isolation of ^{14}C-*p*-bromobenzyl clavulanate *via* the sodium salt (**Ic**)

Harvested culture was adjusted to pH 4.5 with acetic acid and the mycelium removed by centrifugation. The culture supernatant was passed through a column of Amberlite IRA 68 (Rohm & Haas Co., Philadelphia, U.S.A.) anion-exchange resin in the acetate form. The adsorbed clavulanate was eluted with 1.0 M sodium chloride. Eluate fractions containing clavulanate were bulked and reduced to half volume by rotary evaporation *in vacuo*. The concentrate was then passed through an Amberlite XAD-4 column packed in 1 M sodium chloride. The adsorbed sodium clavulanate was eluted with distilled water. Fractions which contained sodium clavulanate and were free of chloride ions were bulked and freeze-dried. The freeze-dried solid was taken up in absolute ethanol and insolubles removed by filtration. The ethanolic solution was evaporated to dryness, and the residue dissolved at 100 mg/ml

in water. Ten volumes of acetone were added to crystallise ^{14}C-sodium clavulanate tetrahydrate (**Ic**). This was converted to **Ia** as in (**1**) above.

Degradations of ^{14}C-*p*-bromobenzyl clavulanate samples

The degradations were carried out as described above for the unlabelled materials. ^{14}C-Labelled **Ia** was normally diluted with unlabelled reference material and recrystallised to constant radioactivity before degradation. Purified degradation products were recrystallised to constant radioactivity. Examination of products by TLC scanning showed only one radioactive component at the appropriate Rf.

Acknowledgements

The authors thank Miss A. STEFANSKA for skilled assistance and Drs. M. COLE and T. T. HOWARTH for helpful discussions.

References

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