The Role of α-Ketoglutarate in Cephalosporin Biosynthesis

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^{13}C Labelled α-ketoglutarates in conjunction with $^{18}O_2$ were used to investigate the role of α-ketoglutarate in cephalosporin biosynthesis.

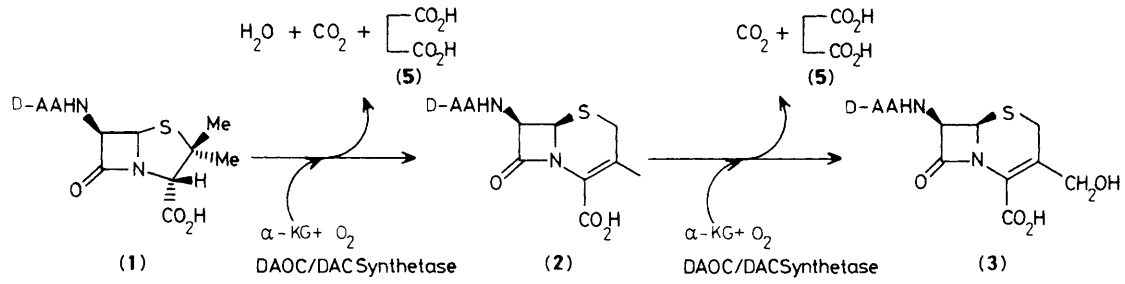
In *Cephalosporium acremonium* the enzymatic ring expansion of pencillin N (**1**) to deacetoxycephalosporin C (**2**, DAOC) and hydroxylation of (**2**) to give deactylcephalosporin C (**3**, DAC) are catalysed by DAOC/DAC synthetase[1] (Scheme 1). Both steps consume dioxygen and α-ketoglutarate (**4**) as cosubstrates and occur with the production of carbon dioxide and succinate (**5**).[2] Recently we have shown that the hydroxycepham (**6**) is a minor product in the ring expansion process[3] and demonstrated that the oxygens of the hydroxy groups of (**6**) and (**3**) were derived at least in part from dioxygen.[3,4]† We now report the results of a study using ^{13}C labelled α-ketoglutarate, designed to reveal the level and regiochemistry of oxygen incorporation into the succinate produced in the enzymatic reactions.

† Previously[4] we have reported levels of incorporation of ^{18}O into DAC (**3**) of 30—40%.

[2-^{13}C]- and [1,2-^{13}C]-α-ketoglutarates‡ were synthesised from $^{13}CH_3I$, as in Schemes 2 and 3, respectively. The key steps in both syntheses were Michael addition of labelled methyl nitroacetates to methyl acrylate,[5] followed by treatment with base and ozonolysis of the resultant nitronates.[6]

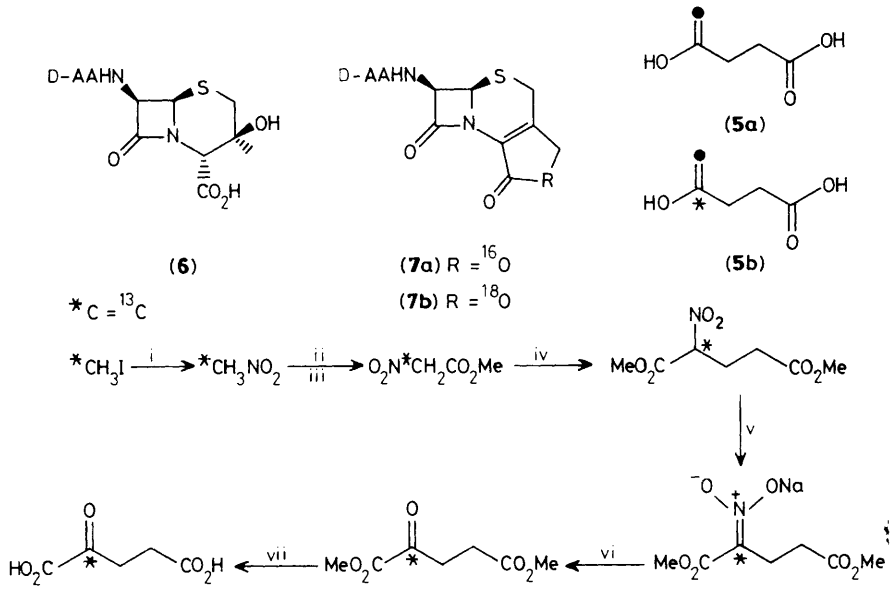
Initially [2-^{13}C]-α-ketoglutarate and DAOC (**2**) were incubated with recombinant DAOC Synthetase.[1b,7] After protein precipitation the incubation mixture was examined by n.m.r. (^1H, 500 MHz; ^{13}C, 125.8 MHz). ^{13}C N.m.r. revealed the signal of [1-^{13}C] enriched succinate [δ_c (125.8 MHz) 182]; which was enhanced by the addition of authentic succinate

‡ The synthesis of the di-labelled material was more efficient, hence 1,2-^{13}C-α-ketoglutarate was used in most experiments. There were no differences observed in the succinate product obtained from mono- or di-labelled α-ketoglutaric acid. Full details of the syntheses will be published elsewhere.[9]

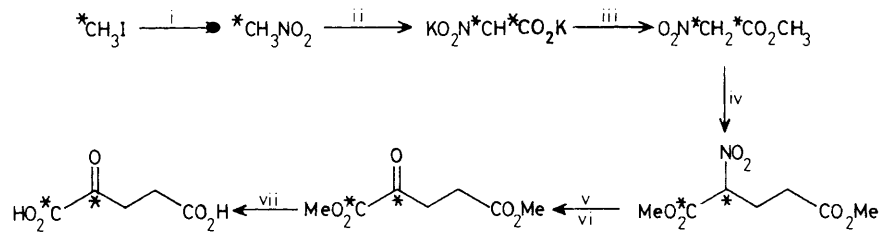


D-AA = δ-(D-α-aminoadipoyl)
α-KG = α-ketoglutarate (4)

Scheme 1



Scheme 2. *Reagents*: i, $AgNO_2/Et_2O$; ii, $MeOCO_2MgOMexCO_2/HCONMe_2$; iii, MeOH/HCl; iv, $PhCH_2(Me)_3NOH/CH_2{=}CHCO_2Me$/dioxane/MeOH; v, NaOMe/MeOH; vi, O_3; vii, HCl/H_2O.



Scheme 3. *Reagents*: i, $AgNO_2/Et_2O$; ii, KOH/H_2O; iii, MeOH/HCl; iv, $PhCH_2(Me)_3NOH/CH_2{=}CHCO_2CH_3$/dioxane/MeOH; v, NaOMe/MeOH; vi, O_3; vii, HCl/H_2O.

(Figure 1). Thus α-ketoglutarate had been converted to succinate. ^1H N.m.r. indicated DAC (**3**) as the sole β-lactam product of the incubation. An analogous experiment with excess penicillin N (**1**) as the β-lactam substrate gave the same conversion of α-ketoglutarate to succinate, concomitant with the formation of (**2**).

We also examined the level of dioxygen incorporation into succinate. An authentic sample of mono-^{18}O labelled succinate (**5a**) was synthesised by reaction of succinic anhydride with $H_2{}^{18}O$. An upfield isotope shift of 0.03 p.p.m. was observed for (**5a**) in accordance with values previously reported for carboxylic acids.[8] Separate incubations of (**1**) and (**2**) with [2-^{13}C]-α-ketoglutarate and degassed DAOC/DAC synthetase under an atmosphere of $^{18}O_2$ gas (96.4%) were carried out. ^{13}C N.m.r. of the incubation mixtures showed a single peak at δ 182 corresponding to mono-^{18}O labelled succinate (**5b**) [confirmed by doping with unlabelled succinate and authentic (**5a**)], indicating >90% incorporation of ^{18}O into

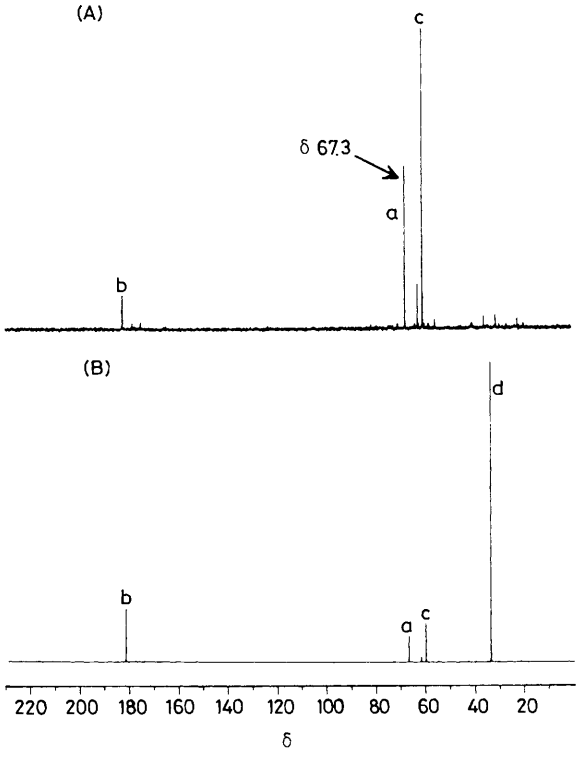


Figure 1. (A) ^{13}C n.m.r. (125.8 MHz) of the crude incubation product after incubation of DAOC (**2**) and [2-^{13}C]-α-ketoglutarate with DAOC/DAC Synthetase under $^{16}O_2$. (B) As (A) but with the addition of *unlabelled* succinate. (a) = dioxane reference, (b) succinate -$^{13}CO_2^-$, (c) Tris buffer, (d) = succinate -CH_2-.

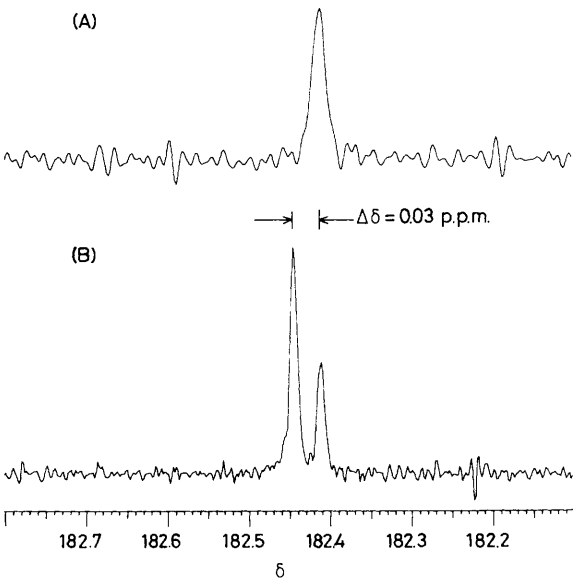
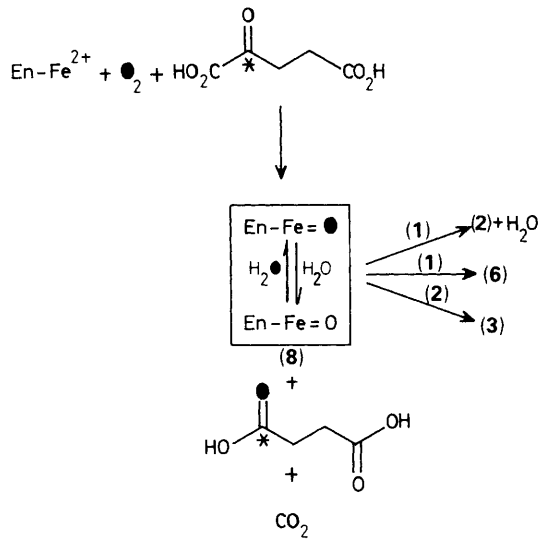


Figure 2. (A) ^{13}C n.m.r. (125.8 MHz) of crude incubation product after incubation of DAOC (**2**) with DAOC/DAC synthetase under $^{18}O_2$. (B) As (A) but with the addition of *unlabelled* succinate.

succinate (Figure 2). From the incubation using DAOC (**2**) as substrate, DAC (**3**) was isolated (reverse phase h.p.l.c., 25 mM ammonium hydrogen carbonate) and lactonised§ to give (**7b**), which was analysed by mass spectrometry (fast atom bombardment)¶ $^{18}O_2$ experiments (*m/z*, % intensity): 355 (6), 356 (86), 357 (27), 358 (100), 359 (24), 360 (10); for (**7a**) derived from a $^{16}O_2$ incubation: 355 (10), 356 (100), 357 (17), 358 (8), 359 (3), 360 (1); calculated for $C_{14}H_{17}N_3{}^{16}O_6S$: 356 (100), 357 (18), 358 (7), 359 (1). This result implies only *ca.* 50% incorporation†† of ^{18}O into DAC (**3**) in contrast with the high level (>90%) of incorporation into succinate (**5b**).



Scheme 4

The stoicheiometry of β-lactam substrate: α-ketoglutarate for the conversions DAOC (**2**) → DAC (**3**) and penicillin N (**1**) → (**2**), respectively, was determined by ^1H and ^{13}C n.m.r. spectroscopy as follows: (i) the amount of β-lactam product formed was determined by integration of the ^1H n.m.r. 6-H and 7-H (β-lactam) signals of the product [(**2**) and (**3**)] against those of the starting material [(**1**) or (**2**)] and a known amount of TSP,§§ (ii) the amount of succinate produced was determined by incubation with [1,2-^{13}C]-α-ketoglutarate and integrations of the ^{13}C n.m.r. signal of the resultant labelled succinate against a known amount of (**5a**) added subsequent to incubation. Both for conversions (**1**) → (**2**) and (**2**) → (**3**), the stoicheiometry of consumption of (**1**) or (**2**): α-ketoglutarate was 1 : 1 (within experimental error ≈ ±10%).

In summary, these results are consistent with a mechanism in which an iron 'oxene' moiety (**8**) is generated by the reaction of enzyme-bound Fe^{2+} with dioxygen and α-ketoglutarate (Scheme 4). Incubation under an atmosphere of $^{18}O_2$ causes each event leading to oxene formation to generate fully mono-^{18}O labelled succinate, stoicheiometrically. Stoicheiometric oxidation of the β-lactam substrates, by the oxene subsequently occurs, *e.g.* (**2**) to (**3**). The less than complete incorporation of ^{18}O into (**3**) is then probably the result of oxygen exchange of the oxene oxygen with water before the substrate oxidation event and is the subject of current investigations.

§ Lactonisation performed with formic acid, ref. 4.

¶ Positive argon ion fast atom bombardment using glycerol as matrix.

†† The possibility that labelled oxygen was lost during the lactonisation event was dismissed by a control experiment in which unlabelled (**3**) was lactonised (HCO_2H) in the presence of $H_2{}^{18}O$ to provide unlabelled lactone (**7a**).

§§ TSP = sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate.

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