# The Role of Molecular Oxygen in Clavulanic Acid Biosynthesis: Evidence for a Bacterial Oxidative Deamination

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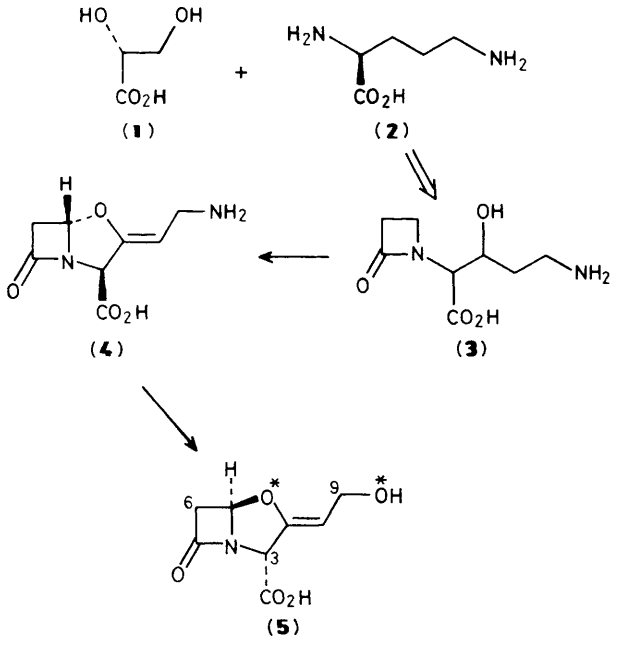
Growth of cultures of *Streptomyces clavuligerus* (ATCC 27064) in an $^{18}O_2$-containing atmosphere resulted in efficient and equal incorporations of molecular oxygen into the oxazolidine ring and the allylic hydroxy group of clavulanic acid.

Oxidative dealkylations at heteroatomic centres are well precedented in natural product biosynthesis.[1] In the case of cytochromes P-450 these processes have received considerable mechanistic attention[2—4] and for a nitrogen centre they are generally believed to involve one-electron oxidation at the heteroatom followed by loss of either a proton or a hydrogen atom from an adjacent C–H bond to give a carbon-centered radical[2,3] or an iminium ion,[4] respectively. This radical or iminium ion is considered to collapse rapidly with the paired iron-hydroxy species at the oxygenase active site to give an



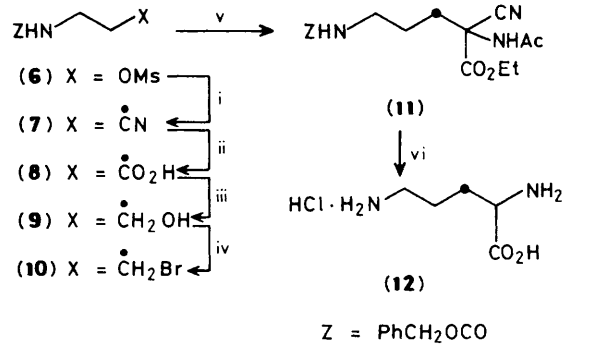
Scheme 1

α-amino alcohol, which is then cleaved to liberate the dealkylated amine. The cognate process of oxidative deamination of a primary amine is virtually unknown.[5] We report here the observation of an apparent case in the course of an investigation to determine the origin of the oxazolidine oxygen in clavulanic acid (**5**).

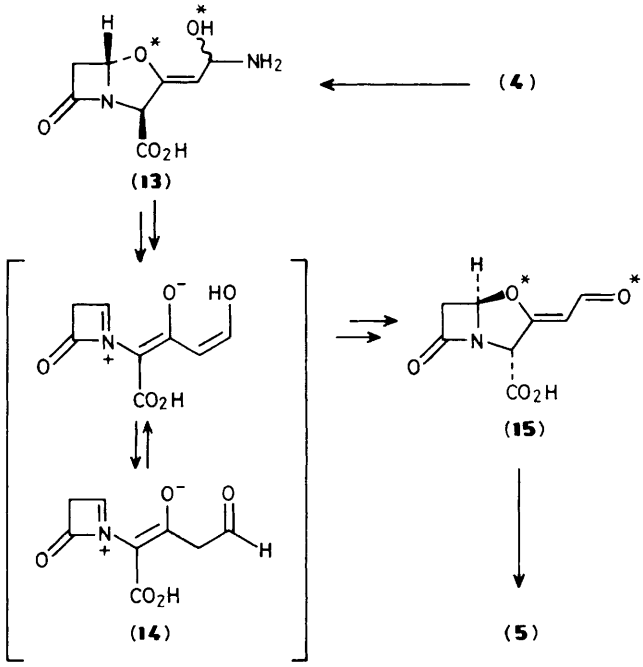
Early reports of Elson[6] pointed to a $C_3$ glycolytic intermediate and an amino acid derived from α-ketoglutarate as the fundamental precursors of clavulanic acid (**5**). Subsequent work provided strong evidence that these building blocks from primary metabolism were D-glycerate (**1**)[7] and L-ornithine (**2**),[8] respectively. An important recent paper reported that proclavaminic acid (**3**) and clavaminic acid (**4**) are intermediates in the formation of (**5**).[9] While the absolute stereochemistry of (**3**) is not known,† clavaminate (**4**) is surprisingly of the configuration antipodal to the biologically active β-lactamase inhibitor clavulanic acid.[10] We have carried out a fermentation of *Streptomyces clavuligerus* (ATCC 27064) under an $^{18}O_2$-containing atmosphere. The recently established intermediacy of clavaminic acid (**4**) now suggests an interpretation of our earlier oxygen incorporation results in which labelled oxygen (*) was found at position 1 and, unexpectedly, at the C-9 OH of clavulanic acid (**5**).

The sites of labelled oxygen incorporation were identified by the now well established technique of monitoring $^{18}O$ isotopic shifts on the n.m.r. signals of the carbon atoms to which the heavy isotope is bound.[11] To enhance the sensitivity of this analysis a sample of DL-[3-$^{13}$C]ornithine (**12**) was prepared in 36% overall yield from the mesylate of 2-aminoethyl *N*-benzylcarbamate (**6**) as shown in Scheme 2, the steps from (**9**) to (**12**) being carried out by a slight modification of Gaudry's procedure.[12] Cultures of *S. clavuligerus* were grown in 12 modified 500 ml shake flasks each containing 100 ml of a glycerol-based fermentation medium[13] essentially as previously described.[8] The flasks were connected in a closed series to a circulating pump with carbon dioxide removal and oxygen replacement carried out essentially as described by Vederas.[14]

† The absolute configuration of (**3**) has been established as L-*threo*, ref. 17.



**Scheme 2.** *Reagents and conditions:* i, 0.36 equiv. K$^{13}$CN, $Me_2SO$, room temp., 120 h, 74%; ii, 2.0 equiv. NaOH in 1:1 MeOH–30% $H_2O_2$ containing 1M NaOH, room temp., 48 h, 97%; iii, 1.1 equiv. $ClCO_2Et/NEt_3$ in THF, −10 °C, 15 min, followed by 4 equiv. $NaBH_4$ 85%; iv, 1.5 equiv. $CBr_4/Ph_3P$, $CH_2Cl_2$, −10 °C, 1 h, 83%; v, vi, see ref. 12.



Scheme 3

At 72 h 1 mmol of DL-[3-$^{13}$C]ornithine was distributed equally among the 12 flasks and, beginning 1 h later, oxygen replacements were made with *ca.* 1:1 $^{16}O_2$–$^{18}O_2$. The clavulanic acid produced was isolated as its *p*-bromobenzyl ester.[8]

$^{13}C\{^1H\}$ N.m.r. analysis of the isolated clavulanate showed a *ca.* 5% incorporation of ornithine as reflected in the enhancement of the C-2 signal. Examination of the resonances assigned to C-2 (δ 152.2), C-5 (δ 88.0), and C-9 (δ 57.3) at high digital resolution (0.12—0.15 Hz) revealed upfield-shifted peaks for each (0.020, 0.029, and 0.016 p.p.m., respectively), indicating a 36 ± 3% incorporation of $^{18}O$ (*) in the oxazolidine ring and at the allylic hydroxyl group of (**5**). The l-oxygen atom and the C-9 hydroxyl group of clavulanic acid, therefore, are derived ultimately from molecular oxygen with apparently equal efficiency. Of the oxidative transformations that elevate ornithine[8] to the level of the $C_5$ unit in clavaminic acid (**4**), it is not known which gives rise to the oxygen present in the oxazolidine ring. Two possibilities would be the ornithyl hydroxylation step that leads to proclavaminate (**3**) and the

α-ketoglutarate-dependent enzymic steps that convert this intermediate into clavaminic acid (**4**). The conversion of (**4**) into clavulanic acid (**5**), which might be thought to involve an amine oxidation or PLP-dependent transamination (PLP = pyridoxal phosphate), proceeds as an oxidative deamination‡ with specific loss of the 9-*pro-S* hydrogen.[15] While initially unexpected, the possibility of involvement of a stable allylic radical[2,3] or conjugated iminium ion[4] *en route* to *e.g.* an α-amino alcohol such as (**13**) favours such a reaction pathway. The remarkable enantiomerization (**4**)/(**13**) could then proceed by one of several detailed routes to the achiral betaine (**14**).[16] Reclosure and release would then give the antipodal aldehyde (**15**), which, as a vinylogous formate ester, may be sufficiently stable to oxygen exchange to be reduced to clavulanic acid without loss of $^{18}O$ label. Alternatively, racemic (**15**) may be the product of this reaction which would be selectively processed by a dehydrogenase to clavulanic acid (**5**).

Professor J. C. Vederas (University of Alberta) is thanked for advice about oxygen incorporations in closed fermentations, and The National Institutes of Health (AI 14937) are gratefully acknowledged for financial support.

*Received, 6th May 1988; Com. 8/017851*

## References

1 K. B. G. Torssell, 'Natural Product Chemistry,' J. Wiley, Chichester, 1983.
2 P. R. Ortiz de Montellano, in 'Cytochrome P-450: Structure, Mechanism and Biochemistry,' ed. P. R. Ortiz de Montellano, Plenum, New York, 1986, ch. 7; P. R. Ortiz de Montellano, *Acc. Chem. Res.*, 1987, **20**, 289.
3 F. P. Guengerich and T. L. MacDonald, *Acc. Chem. Res.*, 1984, **17**, 9.
4 L. T. Burka, F. P. Guengerich, R. J. Willard, and T. L. MacDonald, *J. Am. Chem. Soc.*, 1985, **107**, 2549; see also J. R. Lindsay Smith and D. N. Mortimer, *J. Chem. Soc., Perkin Trans 2*, 1986, 1743.
5 C. J. Parli and R. E. McMahon, *Drug Metab. Disp.*, 1973, **1**, 337; H. Kurebayashi, A. Tanaka, and T. Yamaha, *Arch. Biochem. Biophys.*, 1982, **215**, 433; A. K. Cho and J. Wright, *Life Sci.*, 1978, **22**, 363; T. A. Baillie, *Pharm. Rev.*, 1981, **33**, 81.
6 S. W. Elson and R. S. Oliver, *J. Antibiot.*, 1978, **31**, 586; I. Sterling and S. W. Elson, *ibid.*, 1979, **37**, 1125; S. W. Elson, R. S. Oliver, B. W. Bycroft, and E. A. Faruk, *ibid.*, 1982, **35**, 81.
7 C. A. Townsend and M.-f. Ho, *J. Am. Chem. Soc.*, 1985, **107**, 1066; 3-hydroxypropionate has also been claimed to give rise to the $C_3$ unit: A. L. Gutman, V. Ribon, and A. Boltanski, *J. Chem. Soc., Chem. Commun.*, 1985, 1627.
8 C. A. Townsend and M.-f. Ho, *J. Am. Chem. Soc.*, 1985, **107**, 1065.
9 S. W. Elson, K. H. Baggaley, J. Gillet, S. Holland, N. H. Nicholson, T. J. Sime, and S. R. Woroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1739.
10 S. W. Elson, K. H. Baggaley, J. Gillet, S. Holland, N. H. Nicholson, J. T. Sime, and S. R. Woroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1736.
11 J. C. Vederas, *Nat. Prod. Rep.*, 1987, **4**, 277.
12 R. Gaudry, *Can. J. Chem.*, 1953, **31**, 1060.
13 S. J. Brewer, P. M. Taylor, and M. K. Turner, *Biochem. J.*, 1980, **185**, 555.
14 R. N. Moore, G. Bigam, J. K. Chan, A. M. Hogg, T. T. Nakashima, and J. C. Vederas, *J. Am. Chem. Soc.*, 1985, **107**, 3694.
15 C. A. Townsend, M.-f. Ho, and S.-s. Mao, *J. Chem. Soc., Chem. Commun.*, 1986, 638.
16 For related betaine chemistry, see P. C. Cherry, C. E. Newall, and N. S. Watson, *J. Chem. Soc., Chem. Commun.*, 1978, 469; C. E. Newall, in 'Recent Advances in the Chemistry of α-Lactam Antibiotics,' ed. G. I. Gregory, Royal Society of Chemistry, London, 1981, pp. 151—169.
17 K. H. Baggaley, N. H. Nicholson, and J. T. Sime, *J. Chem. Soc., Chem. Commun.*, 1988, 567; W. J. Krol and C. A. Townsend, unpublished work.

‡ A possible intermediate functionalization of the amine prior to its loss cannot be strictly eliminated, although *a priori* there is no compelling chemical reason for this to occur.