Mb derivatives correspond to negative and positive electron spin densities induced on the meso protons. In the five-coordinate complex where the iron is placed substantially out of the heme and pyrrole nitrogen planes, electron spin may transfer from the iron to the porphyrin through d_{xz}– or d_{yz}–$4e_g\pi^*$(LUMO) orbital mixing, thereby inducing a substantial amount of positive spin on the meso carbon π orbital followed by spin transmission of negative spin onto the meso proton by σ–π spin polarization. In the six-coordinate heme complex, however, the heme iron could move toward the heme planar position, which favors $d_{x^2-y^2}$–σ orbital mixing, inducing positive spin density on the meso proton by spin delocalization mechanism.[19]

In order to further characterize the iron proximal side, we studied ^2H NMR spectra of Mb·^2H_2O, Mb·F^-, and BrCN-modified Mb in ^2H_2O solution. The bulk solvent ^2H_2O signal was suppressed by the conventional WEFT (Water Eliminated Fourier Transform) technique, which has frequently been utilized for the proton NMR measurements in H_2O solution.[21] A single broadened deuterium resonance was observed at around 100 ppm in the far downfield region for above each compound (Figure 1). In comparison with proton NMR of the high-spin monoimidazole–porphyrin complex and with exchangeable proton resonance studies of aquomet Mb in H_2O solution, the deuterium resonance at 100 ppm is readily assigned to the proximal histidyl $N_1{}^2H$. In Mb·F^- and BrCN-modified Mb, the proximal histidyl N_1H proton resonance in H_2O solution was hardly detected and the present ^2H NMR method appears to be very feasible for obtaining the $N_1{}^2H$ resonance. There is no substantial difference in the $N_1{}^2H$ hyperfine shifts between these three high-spin Mb derivatives, implying that presence or absence of the heme iron sixth ligand is not sensed by the proximal imidazole resonances but rather by the heme peripheral meso deuterium resonances. This was also the case for the monoimidazole–porphyrin complex in ferric high-spin state (see Table I). The direct detection of the iron-bound water deuterium resonance was not successful.

We have also tried to observe the meso-deuterium resonances for ferric high-spin HRP reconstituted with meso-$^2H_4$ heme. Unfortunately, no deuterium resonances from meso $^2H_4$ and the proximal $N_1{}^2H$ were observed, although the proximal N^1H proton resonance was seen at 90 ppm in the proton spectrum in H_2O solution.[22] This is probably due to an enhanced quadrupolar relaxation effect on these deuterium resonances for HRP with a molecular weight of 45 000 which has a longer rotational correlation time than Mb (MW = 16 500). Disadvantage of the use of ^2H NMR for the present purpose for large hemoproteins was also experienced for aquometHb. However, *Aplysia* and *Chironomus* Hb's, where the distal histidine is replaced by other amino acid residues and the heme sixth binding site in their ferric high-spin state has been suggested to be vacant from the x-ray and electronic spectral studies,[23,24] would deserve the characterization of the heme iron sixth coordination site by the present meso deuterium NMR method, since these are monomeric and have a molecular weight as much as 16 000.

Registry No. Fe, 7439-89-6; *meso*-$^2H_4$-heme, 94295-13-3; (PPDME)Fe^{3+}(ImH)ClO_4^-, 94295-15-5; (PPDME)Fe^{3+}(2-MeImH)ClO_4^-, 94295-17-7; (PPDME)Fe^{3+}(2-EtImH)ClO_4^-, 94295-19-9; (PPDME)Fe^{3+}(2-*i*-PrImH)ClO_4^-, 94295-21-3; deuterium, 7782-39-0.

(19) In the high-spin $(Me_2SO)_2$ adduct of ferric porphyrin, the heme iron is in the porphyrin plane, in most favor of the σ mechanism. Although the heme iron of aquometMb is displaced from the pyrrole nitrogen plane, the iron displacement as much as 0.27 Å appears to allow the σ-delocalization mechanism. It is therefore likely that the meso proton of the six-coordinate ferric high-spin porphyrin complexes could experience downfield or upfield[20] hyperfine shift depending on the size of the out-of-plane displacement of the heme iron.

(20) Morishima, I.; Kitagawa, S.; Matsuki, E.; Inubushi, T. *J. Am. Chem. Soc.* **1980**, *102*, 2429–2437.

(21) Patt, S. L.; Sykes, B. D. *J. Chem. Phys.* **1972**, *56*, 3182–3184. Inubushi, T.; Ideda-Saito, M.; Yonetani, T. *Biochemistry* **1983**, *22*, 2904–2907.

(22) La Mar, G. N.; de Ropp, J. S.; Smith, K. M.; Langry, K. C. *J. Biol. Chem.* **1980**, *255*, 6646–6652.

(23) Giacometti, G. W.; Ascenzi, P.; Brunori, M.; Rigatti, G.; Giacometti, G.; Bolognesi, M. *J. Mol. Biol.* **1981**, *151*, 315–319.

(24) Steigemann, W.; Weber, E. *J. Mol. Biol.* **1979**, *127*, 309–338.

Biosynthesis of Clavulanic Acid: Origin of the C_5 Unit

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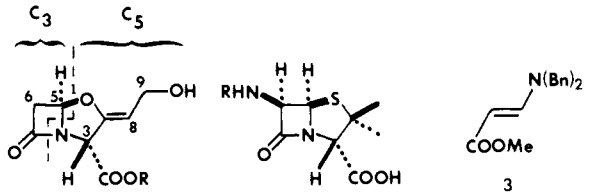
Clavulanic acid (**1**, R = H), a potent inhibitor of bacterial β-lactamases,[1] posesses a fused bicyclic nucleus reminiscent of penicillin N [**2**, R = δ-(D-α-aminoadipyl)] with which it cooccurs



1 2 3

in *Streptomyces clavuligerus*. Preliminary biosynthetic studies by Elson at Beecham[2,3] using doubly ^{13}C-labeled acetate and glycerol and ^{13}C-labeled propionate and bicarbonate indicate that the β-lactam carbons are not derived directly from proprionate but rather from a C_3 intermediate of glycolysis, possibly pyruvate, and that the right-hand portion of clavulanate has its origins in a C_5 amino acid directly related to the TCA cycle intermediate α-ketoglutarate. We present in this communication the results of radiochemical screening and degradation experiments which demonstrate that the precursor of the oxazolidine segment of **1** is ornithine (**7**) rather than δ-hydroxynorvaline (**6**, HNV or 2-amino-5-hydroxypentanoic acid) as has been implied from reported work of the Beecham group with D,L-[2,3-$^{13}C_2$]HNV and -glutamate.[3,4]

Radiolabeled potential precursors of **1** were administered at the concentrations indicated in Table I to vigorously shaken flasks of *S. clavuligerus* (ATCC 27064) after 45–48 h of growth. After a further day and a half, the cells were harvested and **1** was isolated at its *p*-bromobenzyl ester (PBB).[5] The incorporation data obtained are summarized in Table I. That fraction of radioactivity residing in the β-lactam carbons was determined by reaction of **1** (R = PBB) with dibenzylamine in methanol to give the crystalline vinylogous urethane **3**.[2]

The incorporation experiments were conducted using glycerol-[6] or triglyceride-[7]based fermentation media (designated G or T, respectively, in Table I) to control the carbon economy of the cell as was similarly done by Elson.[2,3] The intermediates of glycolysis are generated efficiently from glycerol and metabolism of these to phosphoenolpyruvate and thence acetyl-CoA supplies the TCA cycle from which α-ketoglutarate is withdrawn to generate a C_5 amino acid for clavulanate biosynthesis. Gluconeogenesis from oxaloacetate is effectively suppressed under these carbohydrate-rich conditions. In contrast, the fermentation of triglyceride provides the TCA cycle directly with acetyl-CoA by β-oxidation of fatty acids and gluconeogenesis is a very significant process.

The interesting feature of the C_5 amino acid segment of clavulanic acid is its oxidation state. If one considers oxidation at the β-carbon a later biosynthetic step, then the potential amino

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(1) Hamilton-Miller, J. M. T., Smith, J. T., Eds. "Beta-Lactamases"; Academic Press: London, 1979. Fisher, J.; Belasco, J. G.; Charnas, R. L.; Khosla, S.; Knowles, J. R. *Philos. Trans. R. Soc. London Ser. B* **1980**, *289*, 309–319.

(2) Elson, S. W.; Oliver, R. S. *J. Antibiot.* **1978**, *31*, 586–592. Stirling, I.; Elson, J. W. *Ibid.* **1979**, *32*, 1125–1129.

(3) Elson, S. W. In "Recent Advances in the Chemistry of β-Lactam Antibiotics"; Gregory, G. I., Ed.; Royal Society of Chemistry: London, 1981; pp 142–150.

(4) Elson, S. W.; Oliver, R. S.; Bycroft, B. W.; Faruk, E. A. *J. Antibiot.* **1982**, *35*, 81–86. See also ref 3.

(5) Cf.: Glaxo Laboratories, Ltd., Ger. Offen. 27 08 330.

(6) Small modification of that described in: Brewer, S. J.; Taylor, P. M.; Turner, M. K. *Biochem. J.* **1980**, *185*, 555–564.

(7) Beecham Group Ltd., Ger. Offen. 25 17 316.

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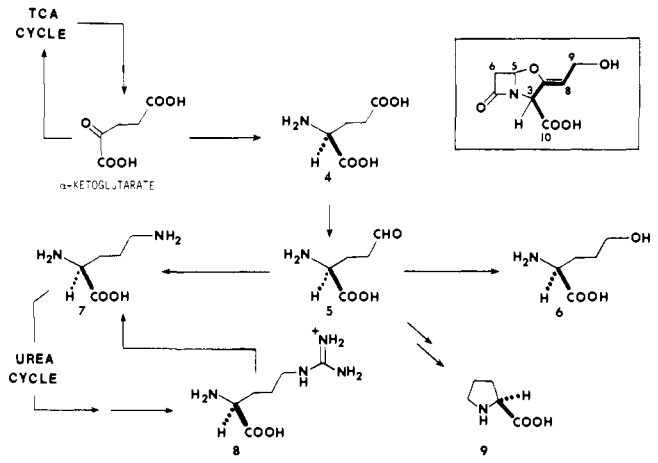
Table I. Incorporation of Radiolabeled Substrates into *p*-Bromobenzyl Clavulanate (**1**, R = PBB)

expt	amino acid	amt,[a] mmol	% ^{14}C spec incorp	% ^{3}H retained	% ^{14}C in C_3 unit	medium[b]
1	L-[U-^{14}C]Glu	0.25	0.24			G
2	L-[U-^{14}C]Glu	0.50	0.32		23.1	T
3	L-[U-^{14}C]HNV[c]	0.25	0.13			G
4	L-[5-^{3}H,U-^{14}C]-HNV[c]	0.40	0.17	15		G
5	L-[5-^{3}H,U-^{14}C]-HNV[c]	0.60	0.54	42		T
6	D,L-[U-^{14}C]-HNV[c]	0.50	0.56		6.0	T
7	L-[U-^{14}C]Pro	0.25	0.11			G
8	L-[U-^{14}C]Arg	0.25	1.61			G
9	L-[U-^{14}C]Orn	0.25	2.11			G
10	L-[U-^{14}C]Orn	0.50	4.86			G
11	L-[U-^{14}C]Orn	0.50	4.01		1.5	T
12	L-[U-^{14}C]Orn	1.00	9.49			G
13	D,L-[5-^{13}H,U,^{14}C]-Orn[c]	0.50	3.69	47		G

[a] Amount of labeled substrate fed (mmol) to 1.5-L total fermentation volume. [b] Principal carbon sources used for two fermentation media: glycerol (G), triglyceride (T), see text. [c] HNV = δ-hydroxynorvaline. Tritium label is stereorandom at C-5.

Scheme I



acid precursors are all readily derived in conventional fashion from L-glutamic acid (**4**) as shown in Scheme I. By administration of 0.25 mmol of **4** and **6**–**9** to fermentations in the glycerol medium (experiments 1, 3, 7, 8, and 9), it can be seen that δ-hydroxynorvaline (**6**, HNV)[8] and proline (**9**) gave similarly low but weakly positive incorporations of radioactivity, glutamic acid (**4**) was about 2-fold higher, and arginine (**8**) and ornithine (**7**) were 15–20 times higher. Comparing relative levels of incorporation in glycerol- and triglyceride-based media for **4**, **6**, and **7** (experiments 1/2, 4/5, and 10/11), with the exception of HNV (**6**), shows that they are quite comparable. Experiments 9, 10, and 12 reveal a nearly linear response of incorporation rate to amount of radiolabeled L-ornithine supplied. In sum these data suggest that among the potential candidates, the urea cycle amino acids, particularly ornithine, are the most efficiently utilized.

The important corollary issue of specificity of labeling may be addressed in several ways. First, **4**, **6**, and **7**, 0.50 mmol of each, were incubated in the triglyceride medium (experiments 2, 6, and 11). As noted above, gluconeogenesis is enhanced under these conditions, and if secondary incorporation were to take place, e.g., by reversion of the C_5 precursor to α-ketoglutarate, circulation of radiolabel around the TCA cycle would result in the appearance of radioactivity in the β-lactam carbons. Degradations of the clavulanate (**1**, R = PBB) isolated in these experiments to (*E*)-methyl 3-(dibenzylamino)acrylate (**3**) afforded the requisite measure of this possibility. For glutamic acid (**4**), HNV (**6**), and ornithine (**7**), the extent of randomization of label into carbons 5–7 was 23.1%, 6.0%, and 1.5%, respectively.[9] Second, the fate of (5*R*, *S*)-[5-^{3}H,U-^{14}C]HNV[6] and -ornithine[10] (experiments 4, 5, and 13) was examined, and notably both of these amino acids lost approximately one-half of their tritium label on incorporation into clavulanate.

At first sight the loss of roughly one-half of the 5-^{3}H label from ornithine and HNV might be interpreted to suggest that glutamate semialdehyde (**5**) provides the logical nexus of the incorporation data, against one's initial structural bias favoring HNV (**6**).[11] While this possibility cannot be strictly excluded, the low specific incorporations of **4**, **6**, and **9** as well as observed randomization of label into carbons 5–7 of **1** in the triglyceride medium disfavor this hypothesis. The urea cycle amino acids are far better utilized, particularly ornithine,[12] with minimum randomization into the C_3 unit of clavulanate. The terminal amino function could be visualized to provide an intermediate binding site for later transamination and reduction.

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Registry No. 1 (R = H), 58001-44-8; **1** (R = PBB), 60297-60-1; **3**, 14376-86-4; **4**, 56-86-0; **5**, 2886-91-1; **6**, 533-88-0; **7**, 70-26-8; **8**, 74-79-3; **9**, 147-85-3.

(8) Murphy, W. H.; Gottschalk, A. *Biochim. Biophys. Acta* **1961**, *52*, 349–360.

(9) An analogous result has been shown for L-glutamate by another approach. See ref 4.

(10) Prepared by considerable modification of the chemistry of: Moe, O. A.; Warner, D. T. *J. Am. Chem. Soc.* **1948**, *70*, 2763–2767. Albertson, N. F.; Archer, S. *Ibid.* **1945**, *67*, 2043–2044.

(11) For comparison a documented role for HNV in polyoxin biosynthesis has been established: Funayama, S.; Isono, K. *Biochemistry* **1977**, *16*, 3121–3127.

(12) The comparatively efficient utilization of ornithine has also been observed at Glaxo (Dr. I. D. Fleming, personal communication).

Biosynthesis of Clavulanic Acid: Origin of the C_3 Unit

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Derivation of the oxazolidine portion of clavulanic acid (**1**, R = H) from a C_5 amino acid, most probably ornithine,[1] maintains a pattern founded in classic experiments of penicillin and cephalosporin biosynthesis[2] and extended in recent studies of nocardicin A[3] and sulfazecin/SQ26180[4] that these β-lactam antibiotics are

† Research Fellow of the Alfred P. Sloan Foundation 1982–1986, Camille and Henry Dreyfus Teacher-Scholar, 1983–1988.

(1) Townsend, C. A.; Ho, M.-F. *J. Am. Chem. soc.*, preceding paper in this issue.

(2) For an excellent review, see: Queener, S. W.; Neuss, N. In "Chemistry and Biology of β-Lactam Antibiotics"; Morin, R. B., Gorman, M., Eds.; Academic Press: New York, 1982; Vol. 3 pp 1–81.

(3) Townsend, C. A.; Brown, A. M. *J. Am. Chem. Soc.* **1983**, *105*, 913–918. Townsend, C. A.; Brown, A. M.; Nguyen, L. T. *Ibid.* **1983**, *105*, 919–927.

(4) Imada, A., paper presented at the 21st Interscience Conference on Antimicrobial Agents for Chemotherapy, American Society for Microbiology, Chicago, IL, Nov 1981. O'Sullivan, J.; Gillum, A. M.; Aklonis, C. A.; Souser, M. L.; Sykes, R. B. *Antimicrob. Agents Chemother.* **1982**, *21*, 558–564.

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