Synthesis of DL-[4-^{3}H]Glutamic Acid for Studies on Clavulanic Acid Biosynthesis

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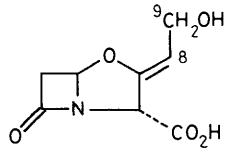
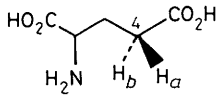
The synthesis of [4-^{3}H]glutamate involved regioselective tritiation of monomethyl succinate, a two-step selective conversion of its free acid function into an aldehyde, and its subsequent conversion by the Strecker reaction into the corresponding α-aminonitrile and hence to the target tritiated glutamate.

Preliminary biosynthetic studies[1] with ^{13}C labelled precursors have shown that the C_5 segment of clavulanic acid (**1**) has its origins in glutamic acid and δ-hydroxynorvaline.[2] Recently, using radioisotope tracer studies, Townsend *et al.*[3,4] have



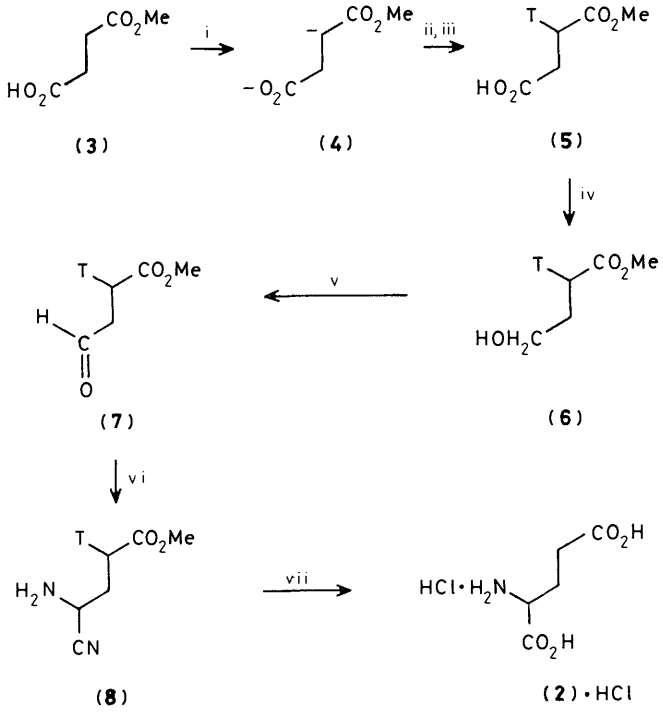
(1)



(2)

indicated ornithine to be particularly well incorporated into the C_5 moiety of clavulanate. For better understanding of the biosynthetic processes leading to the formation of the exocyclic double bond it is necessary to elucidate the origin of the C-8 hydrogen of clavulanic acid. [4-^{3}H]Glutamic acid was used to investigate the possibility that it originates from one of the C-4 hydrogens of glutamic acid (**2**).

The preparation of DL-[4-^{3}H]glutamic acid is illustrated in Scheme 1. The bis anion (**4**) was formed regioselectively by

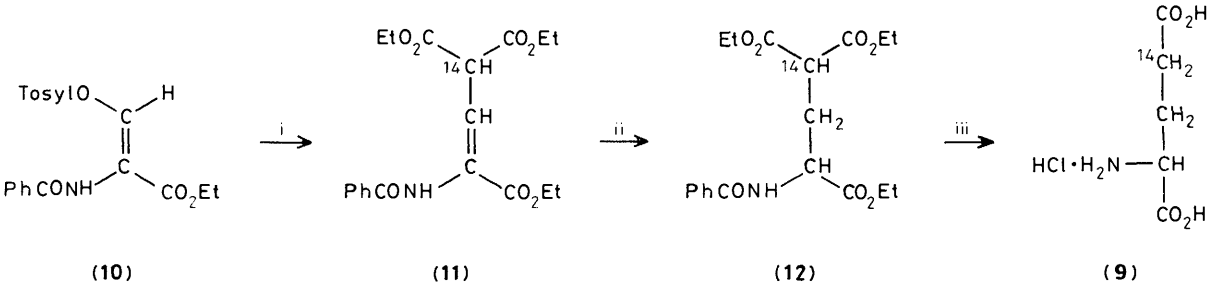


Scheme 1. *Reagents:* i, LDA (2 equiv.); ii, CF_3CO_2T (1 equiv.); iii, H^+; iv, BH_3; v, PCC; vi, KCN/NH_4Cl; vii, HCl/H_2O

reaction of 3-methoxycarbonylpropionic acid (**3**) with two equivalents of LDA (generated *in situ* from butyl-lithium and di-isopropylamine) and quenched with CF_3CO_2T (generated from trifluoroacetic anhydride and tritiated water). Tritium incorporation into (**5**) was significantly higher if, before quenching, most of the regenerated di-isopropylamine was removed by distillation of the solvent at −20 °C under reduced pressure. We assume that di-isopropylamine competes with the bis anion for the added proton to form the di-isopropylammonium cation,[5] which once formed would quench the bis anion by preferential transfer of H^+ rather than T^+, due to the tritium isotope effect. Under those conditions 25—30% of tritium in tritiated water was incorporated into 3-methoxycarbonylpropionic acid to give (**5**). This was converted in 85% yield into the alcohol (**6**) by reaction with borane–dimethylsulphide complex, which is known to reduce acids to alcohols while being inactive towards esters.[6] On treatment with pyridinium chlorochromate,[7] compound (**6**) was oxidized in 80% yield into the corresponding aldehyde (**7**). Treatment of the latter with $KCN–NH_4Cl$ (Strecker reaction[8]) followed by 8 h reflux with 6M HCl and standard work-up afforded in 85% yield the desired glutamic acid hydrochloride (**2**). It is noteworthy that as a result of the reflux with HCl approximately 10% of tritium was lost into the medium, indicating that these harsh hydrolytic conditions would not be suitable for the preparation of C-4 chirally tritiated glutamate.

In order to ensure that the tritium label was introduced selectively on the desired carbon and that it was not lost or scrambled at one of the later steps, it was decided to run through the entire synthesis of glutamic acid with the deuterium label. The regioselectivity of formation of the bis anion (**4**) and of the consequent tritium incorporation into (**5**) was confirmed by quenching (**4**) with CF_3CO_2D to give the deuteriated monomethyl succinate (**5**; T = D). Distribution of deuterium at this stage could not be directly established by ^{1}H n.m.r., since all four methylene hydrogens of (**5**) give a singlet at 2.7 p.p.m. However, n.m.r. examination of the reduced compound (**6**) clearly determined the labelling pattern: the 2.6 p.p.m. triplet, due to the hydrogens α- to the ester function had diminished to about 1.5 H, while the 1.8 p.p.m. multiplet, due to the middle $-CH_2-$, still corresponded to 2 H, but became distorted, because of deuterium coupling. N.m.r. data taken at each of the consecutive synthetic steps showed that deuterium is not lost or scrambled and safely ends up in position α- to the carboxylate amino ester (**8**; T = D) and therefore in position C-4 of glutamic acid. The experiments with deuterium labelling rigorously define the position of the label in glutamic acid and this information should hold good for the synthesis of the tritiated compounds, carried out under identical conditions.

A sample of [4-^{14}C]glutamate (**9**), required as internal standard in the biosynthetic experiments, was synthesized *via* the route shown in Scheme 2. The label was introduced from commercially available diethyl [2-^{14}C]malonate during its condensation with (**10**), prepared according to Bycroft's



(10) (11) (12) (9)

Scheme 2. *Reagents:* i, [2-^{14}C] malonate; ii, H_2/Pd; iii, HCl/H_2O

procedure.[1] Catalytic hydrogenation of (**11**), followed by acid hydrolysis of the saturated triester (**12**) afforded the ^{14}C-labelled glutamic acid hydrochloride (**9**) in 60% overall radiochemical yield (based on diethyl malonate).

The feeding experiments were carried out at Beecham Pharmaceuticals:[9] the tritiated glutamate was mixed with the ^{14}C-labelled glutamate, an accurate ^{3}H:^{14}C ratio was determined, and the mixture was added to shaken cultures of *Streptomyces clavuligerus* during the clavulanic acid production phase. Counting of the isolated and purified *p*-bromobenzyl ester of clavulanic acid indicated that the ^{3}H:^{14}C ratio was reduced to about 9% of that in glutamic acid.

Previously reported work[1] on the incorporation of DL-[3,4-^{13}C]glutamate showed that this precursor undergoes extensive metabolism *via* the TCA cycle and that only *ca.* 13% of the total carbon enrichment of clavulanic acid was due to direct incorporation of glutamate. As only one of the two hydrogens at C-4 of glutamate could be incorporated at C-8 of clavulanic acid then the maximum incorporation of tritium could, therefore, only be about 6—7% of that of the incorporation of the carbon label. Thus the observed retention of 9% of the tritium label is consistent with one hydrogen at C-4 of glutamate being retained during incorporation into clavulanic acid. However, due to the overall incorporation of tritium being low and to the uncertainty as to how much tritium would be lost due to metabolism, this conclusion must be regarded as tentative.

Experimental

Materials and General Techniques.—Diethyl [2-^{14}C]malonate and tritiated water were purchased from Amersham International p.l.c. Radioactive counting involved the use of a Packard liquid scintillation counter, model 526, using [^{3}H]hexadecane as internal standard. ^{1}H N.m.r. spectra were recorded on a Varian T-60 instrument; chemical shifts are reported in p.p.m. downfield from internal tetramethylsilane in $CDCl_3$ unless otherwise noted. I.r. spectra were recorded on a Perkin-Elmer Model 298 spectrometer. Distillations were bulb-to-bulb distillations performed with a Kugelrohr oven at the temperature and pressure indicated. Dry tetrahydrofuran (THF) was obtained by distillation from potassium.

Preparation of [4-^{3}H]*Glutamic Acid Hydrochloride.*—3-*Methoxycarbonyl*[3-^{3}H]*propionic acid* (**5**). Lithium di-isopropylamide was prepared by slow addition of a 1.7M solution of butyl-lithium in hexane (23.5 ml, 40 mmol) to di-isopropylamine (5.7 ml, 40 mmol) in dry THF (70 ml) under argon. The reaction mixture was cooled to 4 °C, stirred for 20 min, and then cooled to −78 °C. 3-Methoxycarbonylpropionic acid (**3**) (2.64 g, 20 mmol) in THF (10 ml) was added to the solution giving a white precipitate. After 1 h at −78 °C the temperature was allowed to rise to about −20 °C and the solvent (70 ml) was distilled out at 0.1 mmHg. Dry THF (50 ml) was added to the mixture, the solvent (50 ml) was distilled out again, and this operation was repeated a further three times. The combined distilled solvent contained 37 mmol of di-isopropylamine, as established by titration. The reaction mixture was solidified by cooling in liquid nitrogen and tritiated trifluoroacetic acid, then CF_3CO_2T was added by vacuum transfer. [The CF_3CO_2T was obtained by vacuum transfer of water enriched with tritium (80 mg, 4.4 mmol, 40 mCi) into trifluoroacetic anhydride (935 mg, 4.4 mmol)]. The reaction mixture was allowed to warm to −40 °C and radioinactive trifluoroacetic acid (8 g, 70 mol) was added. Most of the solvent was evaporated off and the residue was poured into water (30 ml) and extracted with diethyl ether (5 × 30 ml). The combined ether extracts were washed with water (10 ml), dried, and evaporated to afford a yellow oil (**5**) (2.2 g). The material crystallized on standing and was purified to a white solid by trituration with CCl_4. The n.m.r. spectrum was identical with that of pure (**3**), δ 10.9 (s, 1 H, CO_2H), 3.7 (s, 3 H, OCH_3), and 2.6 (s, 4 H, CH_2CH_2). Liquid scintillation counting showed the product to contain *ca.* 10 mCi (25% incorporation of tritium, based on tritiated water).

Methyl 4-*Hydroxy*[2-^{3}H]*butyrate* (**6**).—The foregoing tritiated product (22 mg, *ca.* 100 μCi) was mixed with radioinactive compound (**3**) (283 mg) and the resulting diluted compound (**5**) (305 mg, 2.31 mmol, 105 μCi) was dissolved in dry THF (10 ml). This stirred solution was cooled to −5 °C under argon and a 2M solution of borane–dimethylsulphide complex in THF (1.3 ml, 2.60 mmol) was added dropwise during 10 min. The reaction mixture was stirred for 4 h at −5—0 °C until evolution of hydrogen almost stopped and was then allowed to warm to room temperature overnight. It was then cooled to 4 °C, water (5 ml) was added, and the reaction mixture was stirred for 2 min in order to decompose any remaining borane. Ether (20 ml) was added and the layers separated. The aqueous layer was extracted with diethyl ether (6 × 10 ml) and the combined organic extracts were dried (Na_2SO_4) and evaporated to afford a yellow oil. Bulb-to-bulb distillation gave a colourless fraction of pure (**6**) (230 mg, *ca.* 85 μCi; 85% chemical and radiochemical yield), b.p. 45 °C/0.2 mmHg; δ 3.7 (t, 2 H, *J* 3 Hz, CH_2OH), 3.6 (s, 3 H, OCH_3), 2.8 (s, 1 H, OH, disappears with D_2O), 2.6 (t, 2 H, *J* 3 Hz, CH_2CO_2Me), and 1.7—2.0 (m, 2 H, $CH_2CH_2CH_2$).

Methyl 4-*Oxo*[2-^{3}H]*butyrate* (**7**).—The foregoing compound (**6**) (230 mg, 1.95 mmol, 90 μCi) in CH_2Cl_2 (5 ml) was rapidly added to a stirred suspension of pyridinium chlorochromate (PCC)[6] (530 mg, 3 mmol) in CH_2Cl_2 (30 ml) and the reaction mixture was stirred for 2 h at room temperature. Diethyl ether (60 ml) was added and the black gummy precipitate was separated by filtration through a Florisil bed. Evaporation of the filtrate followed by bulb-to-bulb distillation gave a colourless fraction of pure (**7**) (180 mg, 70 μCi; 76% chemical and radiochemical yield), b.p. 85 °C/1.5 mmHg; δ 9.8 (s, 1 H, CHO), 3.7 (s, 3 H, OCH_3), and 2.4—3.0 (m, 4 H, CH_2CH_2).

[4-^{3}H]*Glutamic Acid Hydrochloride* (**2**).—The aldehyde (**7**) (180 mg, 1.55 mmol, 70 μCi) from the previous experiment was

dissolved in diethyl ether (10 ml), cooled to 4 °C, and treated by stirring with ammonium chloride (93 mg, 1.75 mmol) in water (5 ml) followed by potassium cyanide (104 mg, 1.60 mmol) in water (5 ml). The reaction mixture was stirred at room temperature for a further 4 h, the solvent was evaporated off, and the residue was dissolved in 7M HCl (10 ml) and refluxed for 8 h. Evaporation to dryness afforded a white precipitate which was dissolved in water (10 ml) and extracted with ethyl acetate (10 ml); the aqueous solution was again evaporated. The combined aqueous distillates had *ca.* 10% radioactivity, indicating that some labilization of tritium occurred during the prolonged reflux with 6M HCl. The residue was extracted with methanol (3 × 10 ml) in order to separate the glutamic acid from inorganic residue, and evaporation of the methanol extracts afforded [4-^{3}H]glutamic acid hydrochloride (**2**) (240 mg, 55 μCi; 85% chemical yield, 78% radiochemical yield), as white crystalline material, the n.m.r. spectrum of which in D_2O was identical with that of authentic material.

Deuterium Labelling Experiments.—In order to confirm the position of tritium label in glutamic acid, the synthesis was repeated with deuterium labelling and each stage was monitored by n.m.r. The spectroscopic data of the intermediate products are as follows.

(*a*) 3-Methoxycarbonyl[3-^{2}H]propionic acid (**5**; T = D). This was prepared as described for the tritiated compound: δ 10.7 (s, 1 H, CO_2H), 3.7 (s, 3 H, OCH_3), and 2.6 (s, 3.5 H, CH_2CHD).

(*b*) Methyl 4-hydroxy[2-^{2}H]butyrate (**6**; T = D). This was prepared from the foregoing compound (**5**; T = D) as described for the tritiated compound: δ 3.7 (t, 2 H, *J* 3 Hz, CH_2OH), 3.6 (s, 3 H, OCH_3), 2.6 (m, 1.5 H, $CHDCO_2CH_3$), 2.1 (br s, 1 H, OH, disappears with D_2O), and 1.7—2.0 (m, 2 H, CH_2CH_2CHD). In order to obtain a higher level of deuterium labelling, compound (**5**; T = D) was also prepared by exchange of unlabelled (**5**) with MeOD–MeONa as described in ref. 5. Its reduction with BH_3 as above gave compound (**6**; T = D): δ 3.7 (t, 2 H, *J* 3 Hz, CH_2OH), 3.6 (s, 3 H, OCH_3), 2.6 (m, 0.5 H, $CHDCO_2CH_3$–$CD_2CO_2CH_3$), 2.1 (br s, 1 H, OH), and 1.7—2.0 (m, 2 H, CH_2CH_2CHD).

(*c*) Methyl 4-oxo[2-^{2}H]butyrate (**7**; T = D). This was prepared from the foregoing compound (**6**; T = D) as described for the tritiated compound: δ 9.8 (s, 1 H, CHO), 3.7 (s, 3 H, OCH_3), and 2.4—3.0 (m, 2.5 H, CH_2CHD–CH_2CD_2).

Preparation of [4-^{14}C]*Glutamic Acid Hydrochloride.*—The synthesis of (**9**) was carried out following the route outlined in Scheme 2 by adoption of Bycroft's synthesis of [3,4-$^{13}C_2$]-glutamate.[1] Our modification to that procedure involved recycling of the radiolabelled diethyl malonate, an excess of which had to be used in condensation with (**10**).

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References

1 S. W. Elson, R. S. Oliver, B. W. Bycroft, and E. A. Frauk, *J. Antibiot.*, 1982, **35**, 81.
2 S. W. Elson, in 'Recent Advances in the Chemistry of β-Lactam Antibiotics,' ed. G. I. Gregury, Royal Society of Chemistry, London, 1980, p. 142.
3 C. A. Townsend and M. Ho, *J. Am. Chem. Soc.*, 1985, **107**, 1065.
4 C. A. Townsend, M. Ho, and S. Mao, *J. Chem. Soc., Chem. Commun.*, 1986, 638.
5 A. L. Gutman and J. B. Campbell, *J. Labelled Compd. Radiopharm.*, 1985, **22**, 649.
6 H. C. Brown, S. Krishnamurthy, T. P. Stocky, N. M. Yoon, and C. S. Pak, *J. Org. Chem.*, 1973, **38**, 2786.
7 E. J. Corey and J. W. Suggs, *Tetrahedron Lett.*, 1975, 2647.
8 E. C. Kendall and B. F. McKenzie, *Org. Synth.*, Coll. Vol. 1, 1941, p. 21.
9 S. W. Elson, personal communication.

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