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# SYNTHESIS OF PEPTIDES POTENTIALLY INVOLVED IN THE BIOSYNTHESIS OF CLAVULANIC ACID

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**ABSTRACT**

Two peptides were synthetised by coupling 3-hydroxy propionic acid with L-ornithine and 3-hydroxy-DL-ornithine, to be tested later in order to establish whether they are links in the biosynthesis of clavulanic acid.

Clavulanic acid, produced by *Streptomyces Clavuligerus* NRRL3585[1], is a potent inhibitor of ß-lactamases of gram-positive and gram-negative bacteria[2]. It has a fused nucleus containing a ß-lactam and an oxazolidine ring.(Fig.1).

The biosynthesis of clavulanic acid has been studied by feeding labelled precursors to the fermentation[3,4]. Martin *et al*, showed[5] that ornithine is a clavulanic acid precursor. An ornithine derivative containing a hydroxyl group at carbon 3 appears to condense with D-glyceric acid to form the modified

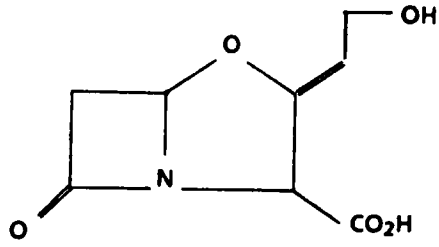
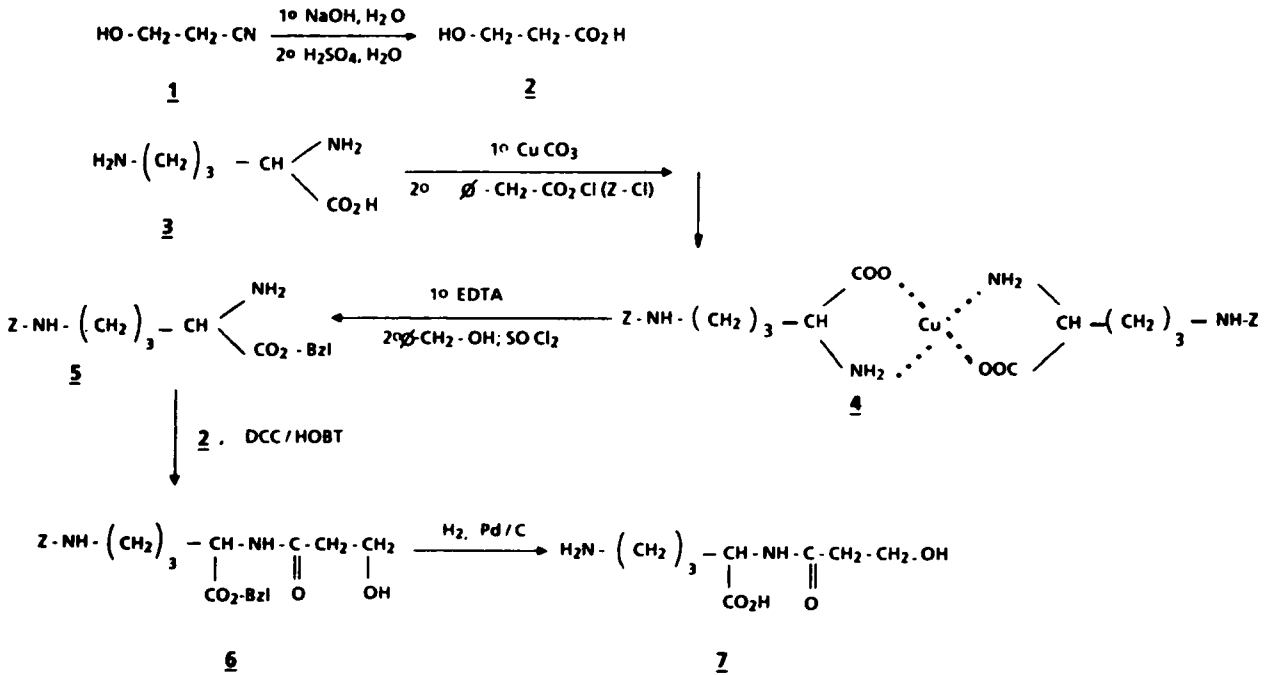



Fig. 1 Clavulanic acid

peptide proclavaminic acid, the first specific intermediate of the clavulanic acid pathway[6]. The hydroxyl group in the peptide would facilitate the formation of the oxazolidine ring of clavulanic acid. This paper describes the synthesis of two peptides which will later be tested in order to ascertain whether they are indeed links in the biosynthetic cycle of clavulanic acid.

These peptides were synthetized by coupling 3-hydroxy propionic acid, with L-ornitine and 3-hydroxy-DL-ornitine. The synthesis of 3-hydroxy propionic acid was carried out using 3-hydroxy propionitrile as a starting point (Scheme 1).

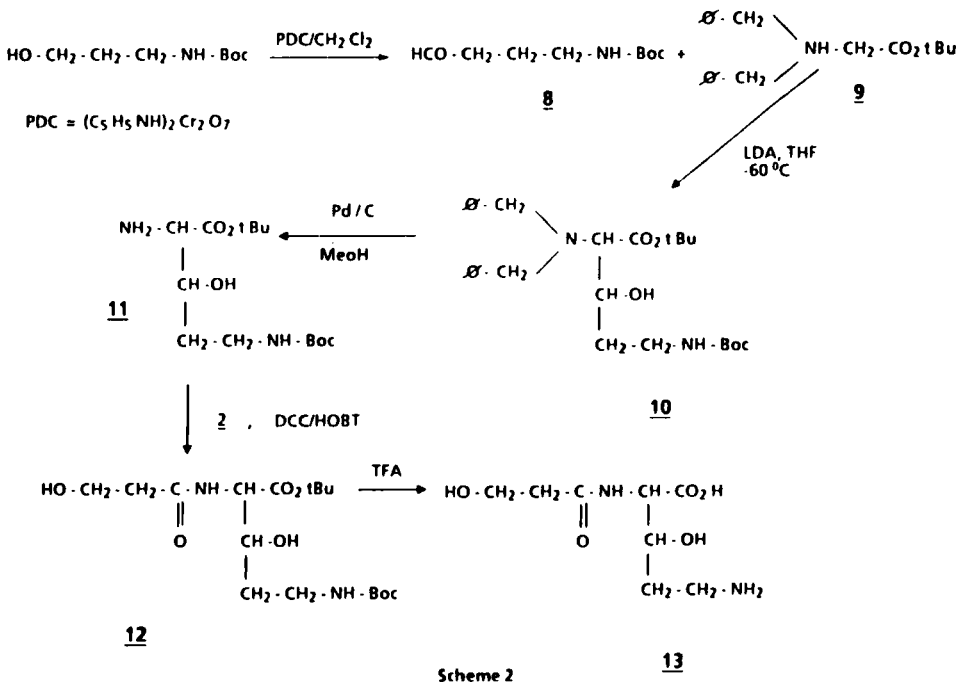
Before bringing about the necessary coupling of L-ornithine with the 3-hydroxy propionic acid in the α-amino group by means of peptide bonding, orthogonal protection must be given to the α-amino and carboxyl groups present in the ornithine, which is achieved by




Scheme 1

blocking their α-amino and carboxyl functions with ornithine's Cu(II) complex[7]. Once this has been done, the δ-amino function is protected using the benzyloxycarbonyl group[8] (Z), after which the complex is removed with EDTA and the carboxylic group is protected with benzyl ester (Bzl). To the ornithine thus protected,3-hydroxy propionic acid is linked through peptide bonding,by means of three activation methods: 1-ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoleine (EEDQ), 1,3-dicyclohexylcarbodiimide (DCC) and DCC/HOBT, (1-hydroxybenzotriazole), the last mentioned giving the greatest yield. After coupling,the product is purified by chromatography and a catalytic reduction[9] is carried out with $H_2$/Pd-C to eliminate protections (Z) and (Bzl).

For the synthesis of the second peptide it was necessary to synthetize the amino acid 3-hydroxy-DL-ornithine first (Scheme 2). Among the various possible ways to construct this α-amino-ß-hydroxy acid, the condensation of a glycine synthetic equivalent with an oxygenated functionality is probably the most straightforward. Our choice was t-butyl-N,N-dibenzyl aminoacetate[10] which can react via an aldol-type condensation[10] with the protected 1-aminopropanal, itself derived from the corresponding aminoalcohol by simple oxidation[11] . The benzyl groups were then cleaved[12] and the resulting product was ready for use.




Scheme 2

It was later coupled by means of peptide bonding with 3-hydroxy propionic acid , using DCC/HOBT, a good yield of the peptide being obtained. These two compounds are currently used in biological experiments as feeding material in the fermentation of *Streptomyces Clavuligerus* in order to ascertain whether the peptides are involved in the biosynthesis of clavulanic acid.

## EXPERIMENTAL

Melting points were determined with a Büchi apparatus in capillary tubes and are uncorrected. The

IR spectra were recorded on a Shimadzu IR-435. The $^1$H-NMR spectra were recorded on Bruker's 80 and 350-MHz instruments with $Me_4Si$ as an internal standard. Microanalyses were performed by the Instituto de Química Orgánica, Madrid.



3-Hydroxy propionic acid (2)

0.7 mmol of 1 was suspended in 100 ml of NaOH-$H_2O$ solution (0.8 mmol), the mixture was stirred at room temperature for 12 hrs. and the solvent removed in vacuo. The $NH_3$ was removed by air suction, heating the flask to 80ºC. To the resultant paste 100 ml of $H_2SO_4$ (0.4 mmol) was added. Then $Et_2O$ was added and the phases separated. The organic layer was dried over $MgSO_4$ and removed in vacuo. Compound 2 was obtained with a 75 % yield. $^1$H-NMR ($CD_3$, $\delta$/ppm): 2.6 (2H,t) ; 3.8 (2H,q) ; 7.6 (2H,s).

Benzyloxycarbonyl-L-ornithine benzyl ester (5)

20 mmol of L-ornithine was dissolved in 80 ml boiling water, to which 200 ml of saturated $CuCO_3$-$H_2O$, and NaOH to pH=8.0 were added, followed by 25 mmol of benzyl chloroformate. The resultant a blue precipitate was filtered and washed with $H_2O$ and EtOH,and dried in vacuo. 4 was obtained with 90% yield.

10 mmol 4 was dissolved in 100 ml of HCl (2N) , to which 30 mmol of EDTA were then added. The mixture was heated under reflux for 1 hr. and the reaction mixture was cooled to 10ºC to obtain a white precipitate Z-δ-L-ornithine, 5, which after vacuo drying was shown to have mp: 265-268 ºC.

10 mmol of 5 was dissolved in 10 ml of benzyl alcohol and 5 ml of $Cl_2SO$ was added. The mixture was stirred at room temperature for 24 hrs. after which excess alcohol was removed in vacuo. The product obtained was suspended in $Et_2O$, and was purified by crystallization with MeOH-$Et_2O$. Compound 5 was obtained with 83% yield. $^1$H-NMR ($Cl_3CD$,δ/ppm) : 1.7 (2H,m) ; 3.2 (2H,t) ; 4.1 (2H,t) ; 4.9 (2H,s); 5.1 (2H,s) ; 7.3 (10 H,s). IR (KBr,$\nu$/$cm^{-1}$) : 3375; 2916; 1650 ; 1584 ; 1516 ; 1233 ; 750 ; 700 . mp=135-137ºC. $C_{20}H_{24}N_2O_4$ Requires : C, 67.41 ; H, 6.74 ; N, 17,98. Found : C, 67.47 ; H, 6.75 ; N, 17.31.

3-Hydroxypropyl-L-ornithine (7)

4 mmol of 5 was dissolved in $CH_2Cl_2$ and chilled to 0ºC. Trietilamine 4 mmol was added, to establish a pH=7.0, which was then checked. 4.4 mmol of 3-hydroxypropionic acid was added followed by 4.4 mmol of HOBT, 10 min. later 4.4 mmol of DCC was added, the mixture was stirred at 0ºC for 4 hrs. and subsequently

for 12 hrs. at room temperature. The reaction mixture was chilled, filtered and evaporated to dryness. The resulting residue was dissolved in ethyl acetate and washed with $H_2O$, HCl (1N), sat. $Na_2HCO_3$ and sat. NaCl solutions. The ethyl acetate layer was dried over $MgSO_4$ and evaporated to dryness. Crude peptide products were purified by silicagel chromatography (9:1-$CH_2Cl_2$/MeOH). Compound 6 was obtained with an 81 % yield. 15 mmol of 6 was dissolved in 40 ml of dry EtOH, 600 mg Pd/C was added and the mixture was stirred for 24 hrs. under $H_2$. The catalyst was filtered out and the solvent was removed in vacuo. Product 7 was suspended in EtOH and purified by crystallization in $Et_2OH$-MeOH. The yield for 7 was 86 % . $^1$H-NMR (250 MHz,$D_2O$,$\delta$/ppm) : 1.7 (2H,m) ; 1.8 (2H,m) ; 2.5 (2H,t) ;3.0 (2H,t) ; 3.8 (2H,t) ; 4.2 (1H,t). IR (KBr, $\nu$/cm$^{-1}$) : 3350 ;3000 ; 1700 ; 1640 ; 1550 ;1400 ; 1250 ; 1050. mp=185-187ºC. $C_8H_{16}N_2O_4$ Requires: C, 47.06 ; H, 7.84 ; N, 13.72. Found : C,47.50 ; H, 7.71 ; N, 13.53.

N-$\delta$-Boc-N$\alpha$-dibenzyl-3-hydroxy-DL-ornithinet-butyl ester (10)

Pyridinium dichromate[11] (46 mmol) was added to a solution of N-protected 3-amino-1-propanol (23 mmol) in 100 ml of methylene chloride. Stirring was continued under $N_2$ for one night. The solution was diluted with ether, filtered and evaporated. Last traces of Cr

species were removed by filtering an ethereal solution through anhydrous magnesium sulfate. The product obtained after evaporation, 8, is highly sensitive and must be used without purification. $^1$H-NMR ($Cl_3CD$, δ/ppm) : 1.4 (9H,s) ; 2.6 (2H,t) ; 3.3 (2H,t) ; 5.2 (1H,m) ; 9.8 (1H,s).

A solution of diisopropylamine (13.9 mmol) in THF (30 ml) was treated at 0ºC under $N_2$ with 1.6 N n-butyllithium in n-hexane (13.5 mmol). After 20 min. the solution was cooled to -60ºC and treated with a solution of N,N-dibenzyl glycine-t-butyl ester (11.6 mmol) in 10 ml of THF. After 10 min. the aldehyde, 8, (11.6 mmol) in 10 ml of THF was added, and after 30 min. the mixture was quenched with satured aqueous $NH_4Cl$, extracted with ether and evaporated to dryness. The crude product was purified by flash chromatography (1:1-ether/hexane) affording pure 10 in 60% yield. $^1$H-NMR ($Cl_3CD$,δ/ppm) : 1.3 (9H,s) ; 1.4 (9H,s) ; 2.8-4.2 (11 H,m) ; 5.0 (1H,m) ; 7.3 (10 H,s).

N-δ-Boc-3-hydroxy-DL-ornithine t-buthyl ester (11)

Ammonium formate (15.0 mmol) was added to a solution of 10 (3.0 mmol) and an equal weight of 10 % Pd/C (1.4 g) in 20 ml of methanol. The mixture was refluxed for 30 min. then filtered and evaporated under reduced pressure to give an oil residue. Chromatography

on silicagel (elution ether) gave 11 in an 87 % yield. $^1$H-NMR ($Cl_3CD$,δ/ppm) :1.4 (9H,s) ; 1.5 (9H,s) ; 1.6 (2H,m) ; 2.6 (3H,m) ; 3.3 (3H,m) ; 3.8 (1H,m) ; 5.1 (1H,m).

3-Hydroxypropyl-DL-3-hydroxyornithine (13)

3.0 mmol of 11 and 3.3 mmol of 2 were coupled by the DCC/HOBT method, as described above. The raw product was purified by silicagel chromatography (95:5-$CH_2Cl_2$/MeOH). The yield for 12 was 82%. For the removal of Boc and tBu protections, 2 mmol of 12 was dissolved in 20 ml of $CH_2Cl_2$-TFA 50%, and stirred for 1 hr. at room temperature then evaporated to dryness. The residues obtained were purified by silicagel chromatography (50:50-$CH_2Cl_2$/MeOH). 13 was obtained as a TFA salt in order to avoid the formation of lactamas. The yield was 91% for 13. $^1$H-NMR (250 MHz,$D_2O$,δ/ppm). 1.2 (2H,m) ;2.1 (2H,m) ; 2.6 (2H,t); 3.2 (2H,m) ; 3.9 (2H,t). $C_8H_{16}N_2O_5$•TFA. Requires: C, 32.40; N, 8.40 ; H, 4.80. Found : C, 34.00 ; N, 9.10 ; H, 4.90.

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