SYNTHESIS OF TRITIATED THREONINE WITH A HIGH SPECIFIC ACTIVITY

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Summary

The synthesis of the trichloromethyl analogue of benzyloxycarbonyl-threonine is described. This precursor was reduced by tritium gas to give [methyl-$^3H_3$]-threonine and its allo isomer. They were separated by HPLC using a chiral stationnary phase. They have a molar specific activity of 3190 GBq/mmol, 86 Ci/mmol.

Key words : selective CrO_3 oxidation, ^3H-labelling, trichloromethylation, trichlorinated Z-threonine.

Introduction

Biological investigations with peptides and proteins frequently require radio-labelled compounds with high specific activities.

Human insulin differs from porcin insulin by only one amino-acid content : threonine replaces alanine at the C-terminal position of the α-chain. We develop an original procedure for labelling the human insulin, which consists of substituing the alanine of the porcine insulin with labelled threonine by the aid of carboxypeptidase.

Catalytic reduction of halogenated or unsaturated precursor by tritium gas is the most convenient way of labelling organic compounds. So we focused our attention on the synthesis of the trichlorinated analogue 7 of Z-threonine. The benzyloxycarbonyl group was chosen as the masking agent of the amino function because of its lability under reductive conditions : the labelling step could directly lead to the free amino-acid. A high specific activity could be expected from the tritiation of the poly-halogenated group.

Results

D- or L-(N-protected)-serines are starting materials in several previously reported asymmetric synthetic routes to respectively L-

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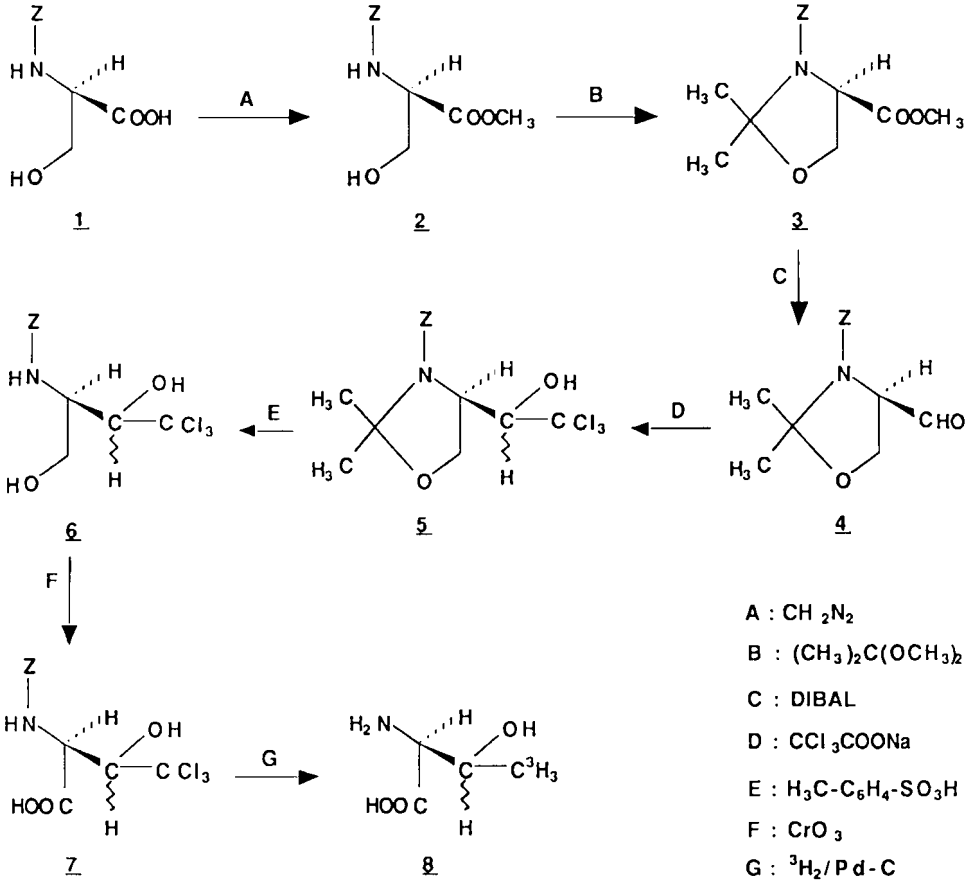
or D-amino-acids (1a-c). They all involved the synthesis of a chiral formyl-oxazolidine. It seems to us that the formyl-oxazolidine 4 could be the target of choice for introducing the trichloromethyl group of compound 7. Indeed, sodium trichloroacetate has been shown to react with aldehydes to give trichloromethylcarbinols (2).

Starting from commercially avalaible Z-D-serine 1, we obtained the formyl-oxazolidine 4, following the procedure (1c) described for its enantiomer. Trichloromethyl anion, resulting from the thermal decomposition of sodium trichloroacetate, reacted in an aprotic solvent (DME) with the aldehydic moiety of compound 4, which then led to the expected product 5.



To open the oxazolidine ring, catalytic quantity of p-toluene sulfonic acid in methanol (1a,c) proved insufficient. At least, 2 equivallents of acid were necessary to obtain the diol 6. Oxidation of the primary alcohol with the Jone's reagent (3a,b) led to the trichloro analogue 7 of Z-threonine. It could be noted that under the chosen reaction conditions, the secondary alcohol was not affected.

The trichlorinated precursor 7 was dehalogenated by tritium gas under pressure with palladium-charcoal as catalyst. Analysis of

the crude [^{3}H]-threonine showed almost the absence of a D-isomer, the relative amount of the two labelled L-diasteoisomers being 20 % for the threonine and 80 % for the allo-threonine.

Experimental

Materials

Z-D-serine was purchased from Bachem and all the reagents used for the synthesis of the cold precursor were obtained fom Aldrich. Reaction evolutions were followed with TLC sheets silica gel 60, revealed with ninhydrine and purifications of the products were made with silica gel 60 (35-70 mesh ASTM), all from Merck. Instruments included a Brücker A-C 270 NMR spectrometer, a Perkin-Elmer 157 G IR spectrophotometer, a Nermag R 10-10 C mass spectrometer, a perkin-Elmer 241 polarimeter and a Perkin-Elmer CHN analyser.

Pure tritium gas was made by the Commissariat à l'énergie atomique (Saclay, France) and distributed by Lumina. The tritium concentration was determined using a Kontron Beta V(liquid scintillation counter). Analytical thin layer chromatography was carried out on Merck Cellulose precoated plates. ^{3}H-scannings of TLC plates were done with a Berthold scanner (Automatic TLC - Linear analyser). The labelled compounds were analysed and purified on a Waters chromatographic system(Chiralpak WH column., Daicel Chemical) and on line with a HPLC radioactivity monitor (Berthold LB 506D). AG50WX12 was purchased from Biorad. Amino acid analysis was carried out on an applied Biosystems amino-acid analyser (130 A Separation system , 420A derivatizer and 920 A Data Analysis module). The catalyst Pd on charcoal was supplied by Fluka. The automatic gas transfer manifold used for catalytic tritiation was previously described (4). The catalyst was separated from the reacting solution by filtration over millipore FG (0.22µm). All solvents were purchased from Prolabo, Merck and BDH. The following abbreviations are used : BAW : n-Butanol-acetic acid-water ; HPLC : high performance liquid chromatography ; TLC : thin layer chromatography ; Z : benzyloxy-carbonyl.

Methods

Precursor synthesis

Compounds **2**, **3** and **4** were prepared according to the procedure of GARNER and PARK (1b).

N-Benzyloxycarbonyl-D-serine, methyl ester **2** : Crude product was purified by column chromatography on silica gel using toluene-ethyl acetate (45-55) as eluent to give the colourless oily compound **2**. Yield: 84 %. ^{1}H NMR ($CDCl_3$) δ 1.82 (1H, br s, exchangeable with D_2O, NH), 3.79 (3H, s, OCH_3), 3.91 (1H, dd, J 11 and 5 Hz, OCH_2), 4.01 (1H, dd, J 11 and 5 Hz, OCH_2), 4.47 (1H, m, CH), 5.15 (2H, s, $ArCH_2$), 5.69 (1H, br s, exchangeable with D_2O, OH) and 7.38 (5H, s, Ar). ν_{max}/Cm^{-1} (KBr) 3600-3200 (OH, NH) and 1760-1680 (CO).

3-Benzyl 4-methyl (R)-2,2-dimethyl-3,4-oxazolidinedicarboxylate **3** : Purification was achieved by column chromatography on silica gel using toluene-ethyl acetate (95-5) as eluent and led to the pale yellow liquid **3**. Yield: 76 %. ^{1}H NMR ($CDCl_3$) δ 1.60 (3H, s, CH_3), 1.73 (3H, s, CH_3), 3.66 (3H, s, OCH_3), 4.15 (2H, m, OCH_2), 4.55 (1H, m, CH), 5.09 (1H, d, J 13 Hz, $ArCH_2$), 5.20 (1H, d, J 13 Hz, $ArCH_2$) and 7.31-7.40 (5H, m, Ar). ν_{max}/Cm^{-1} (KBr) 1760-1670 (CO).

3-Benzyl (R)-4-formyl-2,2-dimethyl-3-oxazolidinecarboxylate **4** : The purification of the product by column chromatography on silica gel using toluene-ethyl acetate (60-40) as eluent gave the yellow oil **4**. Yield: 60 %. $[\alpha]_D$ +41.3 (c 0.57, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 1.58 (3H, s, CH_3), 1.67 (3H, s, CH_3), 4.13 (2H, m, OCH_2), 4.39 (1H, m, CH), 5.13 (2H, s, $ArCH_2$), 7.36 (5H, m, Ar) and 9.56 (1H, s, CHO). ν_{max}/Cm^{-1} (KBr) 2820 and 2720 (CHO) and 1740-1680 (CO).

3-Benzyl (4R)-4-(2, 2, 2-trichloro-1-hydroxyethyl)-2,2-dimethyl-3-oxazolidinecarboxylate **5** : A solution of the aldehyde **4** (17.5 g, 66.5 mmol) and sodium trichloroacetate (14.3 g, 77.2 mmol) in dry dimethoxyethane was heated to 80 °C for 50 mn under argon. The reaction mixture was diluted with 1N HCl aqueous solution and extracted with ethyl ether. Removal of the solvent left a brown viscous liquid which was passed through a column chromatography of silica gel using toluene-ethyl acetate (90-10) as eluent to give the pale yellow oil **5**. Yield: 12 %. $[\alpha]_D$ +7.9 (c 0.31, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 1.58 (3H, s, CH_3), 1.71 (3H, m, CH_3), 3.89 (1H, dd, J 11 and 2.3 Hz, OCH), 4.04 (2H, m, OCH_2), 4.42 (1H, m, CH), 5.16 (1H, d, J 13 Hz, $ArCH_2$), 5.22 (1H, d, J 13 Hz, $ArCH_2$), 7.38 (5H, s, Ar) and 8.05 (1H, d, J 11 Hz, CHOH). ν_{max}/Cm^{-1} (KBr) 3520-3340 (OH) and 1720 (CO). MS (CID, NH_3) *m/z* 405, 403, 401 and 399 $(M+NH_4)^+$, 388, 386, 384 and 382 $(M+H)^+$.

Benzyl (2R)-(4,4,4-trichloro-1,3-dihydroxybut-2-yl)-carbamate **6** : A solution of the oxazolidine derivative **5** (3 g, 7.9 mmol) and p-toluene sulfonic acid monohydrate (17.4 g, 17.6 mmol) in a mixture of methanol-H_2O (70-30) was heated to 65 °C for 1.5 h. Most of the solvent was evaporated under reduced pressure. The suspension was extracted with ethyl ether. After washing the ethereal phase with H_2O, the solvent was then removed. The yellow viscous oil was purified by column chromatography on silica gel using toluene-ethyl acetate (60-40) as eluent. The pure product was recrystallised from toluene to give white crystals of the diol **6**. Yield: 41 %. m.p. 103 °C. $[\alpha]_D$ +0.07 (c 0.27, $CHCl_3$). Found : C, 42.11 ; H, 4.14 ; N, 4.02. $C_{12}H_{14}NO_4Cl_3$ requires C, 42.23 ; H, 4.11 ; N, 4.11. ^{1}H NMR ($CDCl_3$) δ 2.36 (1H, br s, exchangeable with D_2O, NH), 3.89 (1H, dd, J 11 and 2.3 Hz, OCH), 4.36 (1H, m, CH), 4.44 (2H, dd, J 8 and 2.8 Hz, OCH_2), 5.16 (2H, s, $ArCH_2$), 5.83 (1H, d, J 8 Hz, exchangeable

with D_2O, OH), 7.38 (5H, s, Ar) and 7.7 (1H, d, J 11 Hz, exchangeable with D_2O, OH). ν_{max}/Cm^{-1} (KBr) 3440-3260 (OH) and 1730-1670 (CO). MS (CID, NH_3) *m/z* 365, 363, 361 and 359 $(M+NH_4)^+$, 348, 346, 344 and 342 $(M+H)^+$.

2-Benzyloxycarbonylamino-4, 4, 4-trichloro-3-hydroxybutanoic acid 7 : To a solution of the diol 6 (950 mg, 2.8 mmol) in acetone (110 ml) maintained in an ice bath, was added dropwise Jones Reagent (3a) (2.9 ml). Then, the reaction mixture was stirred at room temperature for 3 h. After the reduction of excess of chromium salt with sodium bisulfite, acetone was removed. The aqueous phase was extracted with ethyl acetate. The evaporation of the organic solvent under reduced pressure left the crude oily product, which was purified by column chromatography on silica gel using toluene-ethyl acetate (60-40) as eluent. Recrystallisation from water gave the amino acid 7. (Yield: 32 %) as white crystals. m.p. 100-120 °C. Found : C, 39.17 ; H, 3.37 ; N, 3.64. $C_{12}H_{12}NO_5Cl_3$, 0.5 H_2O requires C, 39.56 ; H, 3.57 ; N, 3.84. ^1H NMR (DMSO-D_6) δ 3.3 (1H, br s, exchangeable with D_2O, NH), 4.05 (1H, d, J 8 Hz, OCH), 4.05 (1H, s, CH), 4.92 (1H, d, J 14 Hz, $ArCH_2$), 5.00 (1H, d, J 14 Hz, $ArCH_2$), 7.28 (5H, s, Ar), 7.49 (1H, d, J 8 Hz, exchangeable with D_2O, OH) and 9.7 (1H, br s, exchangeable with D_2O, COOH). ν_{max}/Cm^{-1} (KBr) 3460-3200 (OH), 2680-2400 (COOH) and 1880-1740 (CO). MS (CID, NH_3) *m/z* 379, 377, 375 and 373 $(M+NH_4)^+$.

^{3}H-labelling of threonine by catalytic dehalogenation

$1.4.10^{-2}$ mmol of threonine precursor 7 was dissolved in 1ml of KOH/CH_3OH (0.1 M) and then frozen. The catalyst (12.3mg, 10% Pd/C) was added and the reacting vial was connected to the tritium gas transfer manifold. When the reacting vial and all tubings had been evacuated (vacuum : 100 Pa), 1295-1480 GBq (35-40 Ci) of pure gas tritium were introduced and compressed at 1.2 10^3 Pa (pressure reduction at room temperature : 21°C). After thawing, the reaction mixture was magnetically stirred for 1.5 h. The absorption of tritium gas produced a pressure reduction of about 18 hPa (at 21°C). By filtration over Millipore, palladium on charcoal was separated from the reaching solution and washed with 20 ml of methanol. Then, labile tritium atoms were eliminated by successive flash rotative evaporations with 2 x 10 ml of pure methanol.

Total radioactivity recovered (crude material) was 4995MBq, 135 mCi. The first attempt to analyse the crude compound was performed using TLC on cellulose (0.22μm) with the solvent system (BAW : 2-1-1). By-products were eliminated using AG 50 WX12 (H^+ form ; diameter :12mm ; lenght : 150 mm) with HCl (increasing concentration). The recovery of the threonine isomeres was effective at 0.75 N and then analytically checked on TLC (cellulose ; BAW : 2-1-1). Enantiomer's purification was performed on a chiralpak WH column (eluent : $CuSO_4$-H_2O, 0.25 M ; temperature : 21°C ; flow rate : 1ml per minute ; U.V. detection : 230 nm). The

analytical radiochromatograms revealed two peaks commigrating with L-allo-threonine and L-threonine (as references) The removal of Cu^{++} was then performed on AG50 WX12 (NH_4^+ form ; diameter : 10mm ; length : 50 mm) in just one simple step (eluent : NH_3-H_2O, 6 M). The [methyl-$^3H_3$]-L-threonine recovered was also checked by TLC (cellulose ; BAW : 2-1-1 ; Rf : 0.35).

Quantitative and qualitative estimations (U.V. spectrophotometric titration, amino -acid analysis , ^3H countings) indicated and confirmed that the specific activity is approximately to be : 3197 GBq /mmol, 86.4 Ci/mmol. Radiochemical purity, greater than 98% was obtained.

After several month of storage in liquid nitrogen, the [methyl-$^3H_3$]-L-threonine retained all chemical and physical properties.

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References

1. a)Garner P.-Tetrahedron Lett. 25: 5855 (1984). b)Garner P. and Park J. M.-J. Org. Chem. 52: 2361 (1987). c)Beaulieu P. L. and Schiller P.-Tetrahedron Lett. 29: 2019 (1988)
2. Winston P., Sharp J. C., Atkins K. E. and Battin D. E.-J. Org. Chem. 32: 2166 (1967)
3. a)Meinwald J. Crandall J. and Hymans W. E.-Org. Synth. 45: 77 (1965). b)Djerassi C., Engle R. R. and Bowers A.-J. Org. Chem. 21: 1547 (1956)
4. Morgat J.L., Demares J. and Cornu M.-J. Labelled Compd. Radiopharm. 11: 257 (1975)