SYNTHESIS OF THE NEW IMMUNOSTIMULATING AGENT PIDOTIMOD (3-L-PYROGLUTAMYL-L-THIAZOLIDINE-4-CARBOXYLIC ACID) LABELLED WITH ^{14}C- AND ^{35}S-ISOTOPES

Patrizia Ferraboschi, Paride Grisenti, Enzo Santaniello
Dipartimento di Chimica e Biochimica Medica, Universita' di Milano
Claudio Giachetti, Giovanni Zanolo
Biomedical Research Institute "Antoine Marxer", RBM
Colleretto Giacosa, Torino
Giovanni Signorelli, Germano Coppi
Research Center, Poli Industria Center, Rozzano, Milano, Italy

Summary
3-L-Pyroglutamyl-L-thiazolidine-4-carboxylic acid (Pidotimod), a new immunostimulating agent, has been prepared labelled with ^{14}C and ^{35}S isotopes starting from L-[U-^{14}C]-glutamic acid **5** and L-[^{35}S]-cysteine hydrochloride **6**, respectively. In the first synthesis, L-[U-^{14}C]-**5** is converted into L-[U-^{14}C]-pyroglutamic acid **2**, which was reacted with ethyl L-thiazolidine-4-carboxylate **3** to afford the ester **4**, in turn hydrolyzed to [^{14}C]-Pidotimod **1**. In the second synthesis, L-[^{35}S]-**6** reacted with formaldehyde to give L-[^{35}S]-thiazolidine-4-carboxylic acid **7**, which was coupled with the activated ester of pyroglutamic acid, compound **8**, to afford [^{35}S]-Pidotimod **1**. The total activity of [^{14}C]-Pidotimod was 1.2 mCi (specific activity 5.52 mCi/mmol) and that of [^{35}S]-Pidotimod was 1.0 mCi (specific activity 9.43 mCi/mmol).

Key words: [^{14}C]-Pidotimod; [^{35}S]-Pidotimod; L-[U-^{14}C]-pyroglutamic acid; L-[^{35}S]-cysteine; immunostimulating agent.

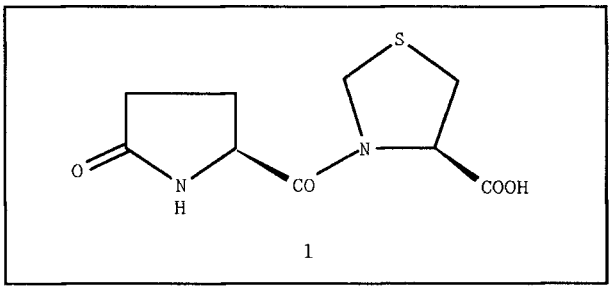
Introduction

Biological response modifiers have a rationale for use in primary or acquired immunodeficiences and recurrent infections diseases may represent a field of application for immunostimulating agents. In fact, a relative or absolute decrease in immune response has been reported in recurrent infections diseases both in children, such as respiratory or urinary infections, and in adults, such as chronic obstructive pulmonary disease. Biological response modifiers commercially available from extract sources pose two problems: first, purity and reproducibility of the pharmaceutical preparations; second, oral bioavailability. Pidotimod [3-L-pyroglutamyl- L-thiazolidine-4-carboxylic acid, compound **1**] is

0362–4803/92/120973–08$09.00

Received 3 June, 1992
Revised 14 August, 1992

the first compound of a new class of biological response modifiers with a peptide-like structure, synthetic origin and oral bioavailability, which increases cell-immediate immune response by stimulation of IL-2 production.[1-5]



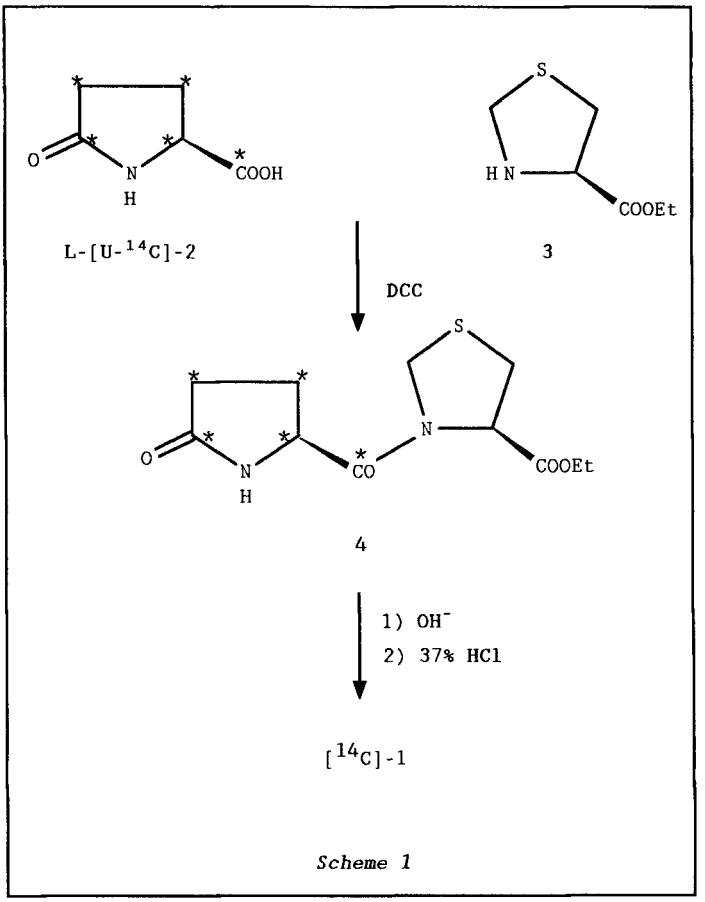
1

For studies of pharmacokinetics and metabolism, samples of Pidotimod **1** labelled with radioactive isotopes were required. The original plan was to use [^{14}C]-Pidotimod, which could be prepared from the corresponding amminoacids labelled with ^{14}C, with a minimum requirement of 1 mCi total activity in 50 mg of the final compound. However, one limiting factor was the fact that neither ^{14}C-cysteine or ^{14}C thiazolidine-4-carboxylic acid of sufficient activity were available. For this reason, we decided to prepare two samples with the same total activity, one labelled with ^{14}C at the pyroglutamyl moiety of the dipeptide and the other with ^{35}S at the thiazolidine moiety.

Synthesis of [^{14}C]-Pidotimod 1

The synthetic route followed for the preparation of [^{14}C]-**1** was in accord with the method used for the production of the compound on industrial scale[6] (Scheme 1). However, some problem was encountered in the use of L-[U-^{14}C]-pyroglutamic acid **2** as furnished by Amersham International, U.K. (1 mCi total activity, specific activity 18.4 mCi/mmol). The required compound **2** was available as an aqueous solution and for the DCC-mediated coupling[7] with the ethyl thiazolidine-4-carboxylate **3**, the labelled pyroglutamic acid **2** should be perfectly anhydrous.

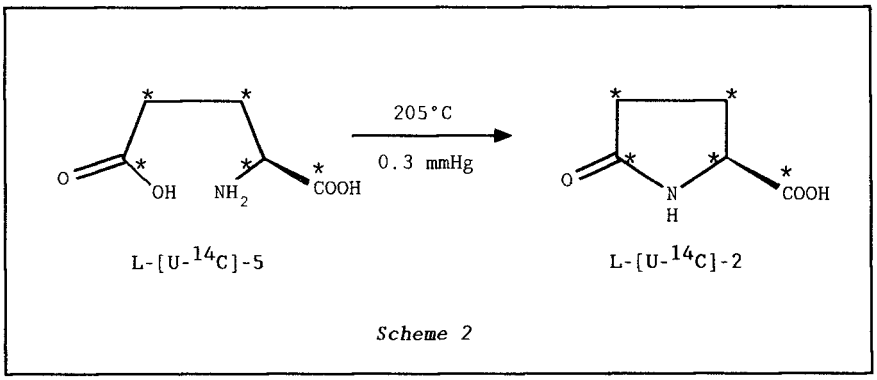
An excess of N,N-dicyclohexylcarbodiimide (DCC), necessary for the



Scheme 1

synthesis, would add complications for the purification of the coupling product **4**. For this reason, after addition of unlabelled **2** (50 mg) to the commercial solution of L-[U-^{14}C]-**2**, it was necessary to lyophilize the resulting labelled sample. At this point, probably some opening of the pyrrolidinone ring was a consistent side reaction. In fact, alkaline hydrolysis of the ethyl ester **4**, followed by acidification afforded [^{14}C]-**1** (20 mg) of total activity 0.24 mCi (specific activity 2.9 mCi/mmol). The aqueous solution from the work-up of the DCC reaction contained a total activity of 0.7 mCi, from which the product of hydrolysis of **2**, L-[U-^{14}C]-glutamic acid, could be recovered by exchange resin chromatography. Therefore, for our preparative purposes, we decided

to prepare labelled **2** by a classical dehydration-sublimation of L-[U-^{14}C]-glutamic acid **5**[8] (Scheme 2).



L-[U-^{14}C]-5 L-[U-^{14}C]-2

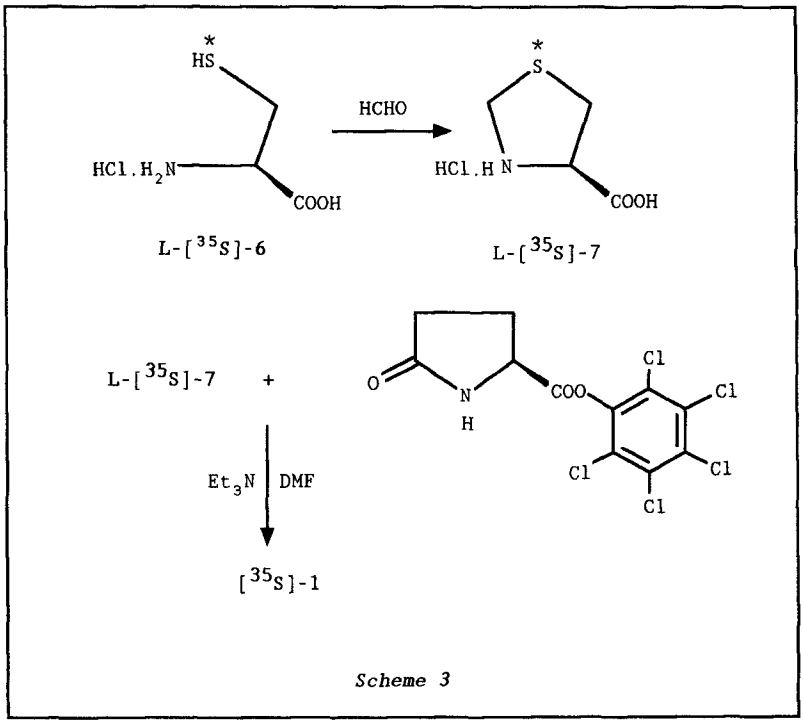
Scheme 2

Starting from a commercial sample of L-[U-^{14}C]-**5** with a specific activity of 250 mCi/mmol (5 mCi total activity), L-[U-^{14}C]-pyroglutamic acid **2** could be prepared in 86% chemical yield (3 mCi) when the reaction was carried out at reduced pressure (0.3 mm Hg) and a temperature of 205 °C. For the preparative use, 50 mg of labelled Pidotimod **1** was needed with a total activity of 1 mCi and therefore the required L-[U-^{14}C]-glutamic acid **5** (5 mCi) was diluted with unlabelled glutamic acid (50 mg) and processed as described in Scheme 2. The sample of L-[U-^{14}C]-pyroglutamic acid **2** (84 mg, 3 mCi total activity) was used for the next step, namely the DCC-mediated condensation with the unlabelled ethyl L-thiazolidine-4-carboxylate **3** and the ethyl ester **4** was directly hydrolyzed to the final dipeptide **1**. The [^{14}C]-**1** was obtained in 70% chemical yield from labelled **2** as a crude product of the reaction and was diluted with 20 mg of unlabelled Pidotimod **1** and crystallized twice to obtain the pure product with constant radioactivity (total activity 1.2 mCi, specific activity 5.52 mCi/mmol).

Synthesis of [^{35}S]-Pidotimod 1

Starting from L-[^{35}S]-cysteine hydrochloride **6**, we prepared

L-[^{35}S]-thiazolidine-4-carboxylic acid **7** and the ethyl ester **3** from it, in order to follow the route depicted in Scheme 1. Due to some experimental problem encountered in the microscale conditions, we were unable to perform the coupling of the ester **3** with unlabelled pyroglutamic acid **2**. Thus a new approach was developed for the synthesis of [^{35}S]-**1** (Scheme 3).



Scheme 3

L-[^{35}S]-Thiazolidine-4-carboxylic acid hydrochloride **7** (100 mg, 2.1 mCi total activity) was prepared from L-[^{35}S]-cysteine (5 mCi, 106 mCi/mmol specific activity) and formaldehyde.[9] The compound **7** was coupled (Et_3N, dimethylformamide) with an activated ester of pyroglutamic acid, namely pentachlorophenyl pyroglutamate **8**, prepared according to a known method[10] to afford [^{35}S]-**1** (26 mg, 35%) of total activity 1 mCi (specific activity 9.43 mCi/mmol).

Experimental

Unlabelled ethyl thiazolidine-4-carboxylate was prepared by reaction of thiazolidine-4-carboxylic acid with thionyl chloride and ethanol.[6] L-[U-^{14}C]-pyroglutamic acid (1 mCi, with a specific activity of 18.4 mCi/mmol, 680 MBq/mmol, 99% radiochemically pure), L-[U-^{14}C]-glutamic acid (5 mCi, with a specific activity of 250 mCi/mmol, 10 GBq/mmol, 98.7% radiochemically pure), and L-[^{35}S]-cysteine (5 mCi, 106 mCi/mmol specific activity, 3,922 MBq/mmol, 96% radiochemically pure) were purchased from Amersham International, UK, and used without purification. Hplc analyses were carried out on a Jasco (Japan Spectroscopic Co., Ltd) apparatus using a LiChrosorb 5 μm amino column (4.0mm x 20cm) eluting with phosphate buffer, $(NH_4)H_2PO_4/H_3PO_4$ 0.01M, pH 3): acetonitrile 97:3 v/v at 1 ml/minute, UV detection at 210 nm. Radiochemical purity was assayed by tlc on silica gel 60 F254 plates (0.25 mm layer, Merck, Darmstadt, D). The elution systems used were: system a, $CHCl_3$:CH_3OH:CH_3COOH (60:30:10, v/v); system b, $CHCl_3$:CH_3OH:H_2O:NH_4OH (38:30:7.5:7.5, v/v); system c, $CHCl_3$:CH_3OH:CH_3COOH:H_2O (40:40:10:20, v/v). Tlc autoradiographs were recorded on a Bioscan Imagin Scanner (Canberra Packard, Milan, I). The radioactive material co-chromatographed with authentic unlabelled compound in all the above chromatographic systems. Radiochemical purities were found 98.1 and 98.7% [a], 97.8 and 98.2% [b], 98.3 and 98.5% [c] for the ^{14}C and ^{35}S labelled compounds, respectively. Radioactivity countings were performed on a Tricarb 2000A Counter (Canberra Packard, Milan, I) with external standard quenching method (tSIE), carried out using the analytical hplc system above. Liquid scintillation counting was performed using a Beckman LS6800 counter. Mass spectra were registered on a Hewlett Packard Instrument Mod.5988, by direct inlet probe and electronic impact (electron energy at 70 eV and ion source at 270°C) techniques.

L-[U-^{14}C]-Pyroglutamic Acid 2.- L-[U-^{14}C]-glutamic acid (aqueous solution containing 2% ethanol; 5 mCi; 250 mCi/mmol specific activity) were diluted with an aqueous solution of unlabelled glutamic acid (50 mg, 0.34 mmol) and evaporated to dryness (40 °C, 0.1 mmHg) in 4 hours. The solid obtained was sublimed at reduced pressure (205 °C, 0.3 mmHg) and pure L-[U-^{14}C]-pyroglutamic acid **2** was obtained (38 mg, 0.29 mmol, 3 mCi). M.S.: *m/z* 130 $[M+1]^+$; 85 $[M-44]^+$. Tlc: $CHCl_3/CH_3OH/CH_3COOH/H_2O$ = 75/60/15/15.

[^{14}C]-Pidotimod 1.- In dry dichloromethane (7 mL) solid $NaHCO_3$ (0.45 g) and ethyl thiazolidine-4-carboxylate hydrochloride **3** (1.125 g, 5.69 mmol) were added and the mixture was stirred at room temperature for 12 hours, then the reaction was evaporated to dryness at reduced pressure (40 °C, 16 mmHg). Dichloromethane (10 ml) was added and from the solution an aliquot was taken (509 μl) and added to the previously prepared L-[U-^{14}C]-pyroglutamic acid **2** (38 mg, 0.29 mmol). To the reaction, cooled to 0 °C, N,N-dicyclohexylcarbodiimide (DCC, 60 mg; 0.29 mmol) was added. The reaction was stirred at 0 °C for 1 hour and at room temperature for one additional hour, then the mixture filtered off

and the precipitate washed with dichloromethane (0.5 ml). The resulting solution was concentrated under nitrogen to 0.5 ml and, with the temperature at 0 °C, a solution of NaOH (12 mg) in water (100 µl) was added. The reaction was kept stirring at room temperature for 30 min, then the aqueous phase was separated and acidified (pH 2) with HCl (37%, 30 µl). This solution was kept at 0 °C for 1 hour and the solid so obtained recovered by filtration and washed with cold water (2 x 100 µl) The crude product (50 mg) was diluted with 20 mg of unlabelled PGT 1/A and crystallized twice from 2-propanol-water (4:6, 0.5 mL) to obtain the pure product [^{14}C]-**1** with constant specific radioactivity (53 mg, 1.2 mCi,). M.S.: *m/z* 244 $[M]^+$, 226 $[M-18]^+$, 199 $[M-45]^+$. Tlc: $CHCl_3/CH_3OH/NH_3/H_2O$ = 75/60/15/15.

L-[^{35}S]-Thiazolidine-4-carboxylic Acid Hydrochloride 7.- An aqueous solution of L-[^{35}S]-Cysteine hydrochloride **6** (7.4 mg in 280 µl of water) was poured in a round-bottom flask containing 7N hydrochloric acid (0.5 mL). Unlabelled cysteine hydrochloride (42.6 mg, 0.27 mmol) was added and the solvent evaporated under nitrogen. The diluted cysteine hydrochloride (0.31 mmoles) was dissolved in 50 µl of water and 7N hydrochloric acid (50 µl). An aqueous solution of formaldehyde (36%, 32 µl, 0.42 mmoles) was added and the mixture was kept at room temperature overnight. The pH of the solution was brought to 4.5 with 0.7N NaOH and the product **7** (47 mg) precipitated at 4°C after concentration of the solution under nitrogen. An aliquot of the precipitated **7** was counted with liquid scintillation and a total radioactivity of 2.1 mCi was determined.

[^{35}S]-Pidotimod 1.- A solution of the above hydrochloride **7** (45 mg, 0.298 mmoles) in dimethylformamide (0.8 mL) was treated with triethylamine (74µl) and the pyroglutamic ester **8** (112 mg, 0.298 mmol). The solution was kept stirring at room temperature (24 hours) and, after evaporation at reduced pressure, water was added (1.5 mL). The aqueous solution was extracted with diethyl ether (3 x 1 mL) and brought to pH 2 with 6 N HCl. The aqueous solution was concentrated under nitrogen to 0.5 mL and kept at 4 °C. The precipitate was filtered and recrystallized twice from 2-propanol-water (4:6), to obtain pure [^{35}S]-**1** (26 mg, 1 mCi) .

Acknowledgwments.
We thank Mr. Andrea Lorenzi for the mass spectra and Miss Elisa Verza for skillful experimental assistance.

References

1. M. Barchielli and G. Coppi *Europ. J. Pharm. 183*, 909 (1990).
2. G. Coppi and F. Mailland *Pharmacol. Res. 22* (Suppl. 2), 126 (1990).
3. M. T. Illeni, G. Bombelli and G. Sciorelli *Proceed. Second Refresher Course of Immunohaematology*, Rieti (Italy), June 21-23, 1989.
4. P. L. Meroni, F. Capsoni, W. Barcellini, F. Minonzio and C. Zanussi *5th International Conference on Immunopharmacology* Tampa (Florida, USA), May 26-30, 1991.
5. A. Auteri, A. L. Pasqui, F. Bruni and M. Saletti *Symposium on Standardization of the Immunopharmacology of Natural and Synthetic Immunomodulators* (Satell. Symp. of the 5th Int. Conf. of Immunopharmac.) Annecy (France), May 15-17, 1991.
6. S. Poli and L. Del Corona *European Patent* (Appl. n. 90119271-6, 1990).
7. N. F. Albertson *Org. Reactions, 12*, 205 (1962).
8. R. Brenner and H. R. Riekenbacher *Helv. Chim. Acta, 41*, 181 (1958).
9. S. Ratner and H. T. Clarke *J. Am. Chem. Soc. 59*, 200 (1937).
10. G. Flouret *J. Med. Chem. 13*, 843 (1970).