SYNTHESIS OF N-[N-L-γ-GLUTAMYL-L-CYSTEINYL-(CARBONYL-^{14}C)]-GLYCINE (GLUTATHIONE-^{14}C) AND OF [CYS-^{14}CO]-(5S,6R)-LTC$_4$

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SUMMARY

[Cys-^{14}CO]-glutathione or N-[N-L-γ-glutamyl-L-cysteinyl-^{14}CO] glycine was prepared by chemical synthesis, with the L,L-natural configuration and a radiochemical yield of 1 % based on sodium [^{14}C] cyanide. This labelled compound was used to obtain [Cys-^{14}CO] with a yield of 15 %. [Cys-^{14}CO]-glutathione and [Cys-^{14}CO]LTC$_4$ had a specific activity of 50 mCi/mmol (1.85 GBq/mmol).

Key words : peptidoleukotrienes, (D,L)- and (L,L)glutathione-^{14}C, leukotriene C$_4$-^{14}C.

INTRODUCTION

The stereo- and enantiospecific enzymatic oxidation of arachidonic acid 1 by 5-lipoxygenase leads to peptido leukotrienes (5S,6R)-LTC$_4$ 3, (5S,6R)-LTD$_4$ 4 and (5S,6R)-LTE$_4$ 5, through the intermediate unstable allyl epoxide : (5S,6S)-LTA$_4$ [1] 2 (scheme 1). As LTC$_4$ is the biogenetic precursor of LTD$_4$ and LTE$_4$, it is rapidly metabolized by γ-glutamyl transpeptidase and cysteinyl glycinase successively [2]. These peptidolipids have high biological activities like contraction of smooth muscles or vasodilatation [3]. Thus, they are very active mediators involved in immmediate hypersensitivity reactions and asthma related diseases [4]. The study of peptidoleukotrienes biosynthesis and metabolism is particularly interesting.

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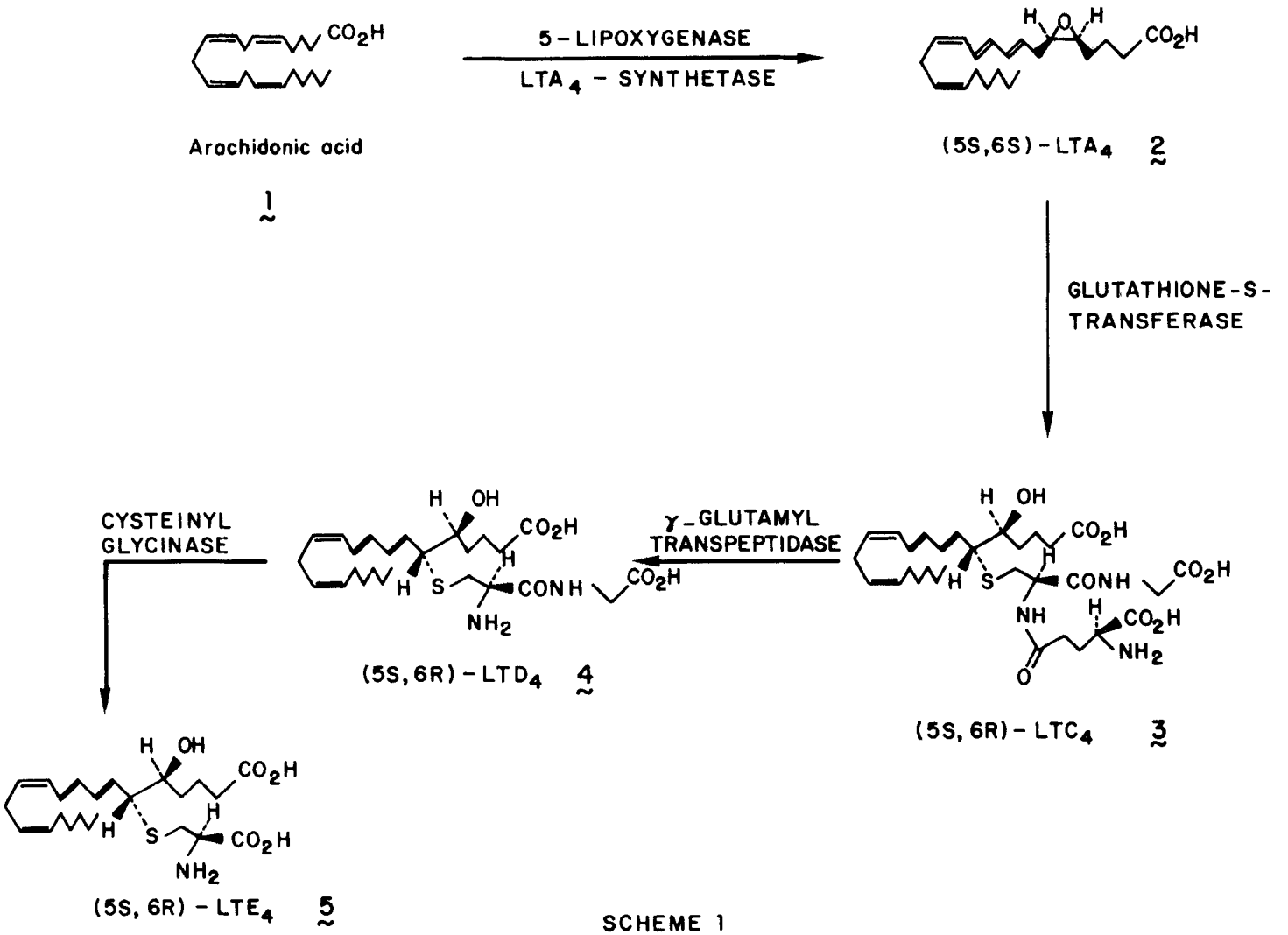

5-LIPOXYGENASE
LTA4 - SYNTHETASE
Arachidonic acid
1
(5S,6S)-LTA4 2
GLUTATHIONE-S-TRANSFERASE
γ-GLUTAMYL TRANSPEPTIDASE
CYSTEINYL GLYCINASE
(5S,6R)-LTD4 4
(5S,6R)-LTC4 3
(5S,6R)-LTE4 5

SCHEME 1

Herein, a chemical synthesis of [Cys-^{14}CO]LTC$_4$ 21 is described (schemes 2 and 3). The labelling position was chosen in relation with the rapid metaboslism of LTC$_4$ into LTE$_4$ and also with the β-oxidation of the lipophilic chain of LTC$_4$ (C_{12}-C_{20}), which reaction was recently demonstrated in incubating LTE$_4$ with rat hepatocytes [5].

Contrary to the tritium labelling of the lipophilic part of LTA$_4$ ([11, 12, 14, 15-^{3}H]-LTA$_4$ methyl ester [6]) and LTE$_4$ ([14, 15-^{3}H] -LTE$_4$ [7]), the labelling of the glutathionyl residue of LTC$_4$, to our knowledge, has not yet been described in the literature.

Several enzymatic methods of synthesis of labelled glutathiones are described : glutathione-^{35}S [8] or [Glu-U-^{14}C] glutathione [9]. However, these labelled molecules have some disadvantages :

- very low specific activities : 0,6 mCi/mmol for [Glu-U-^{14}C]-glutathione and 0.66 mCi/mmol for glutathione-^{35}S.
- the labelling position in glutathione-^{14}C in the [U-^{14}C-glutamyl]-residue, is not appropriate for some biological studies.
- these compounds require advanced techniques such as a genetic engineering in order to produce cells enriched in glutathione synthetase and γ-glutamyl-cysteine synthetase.

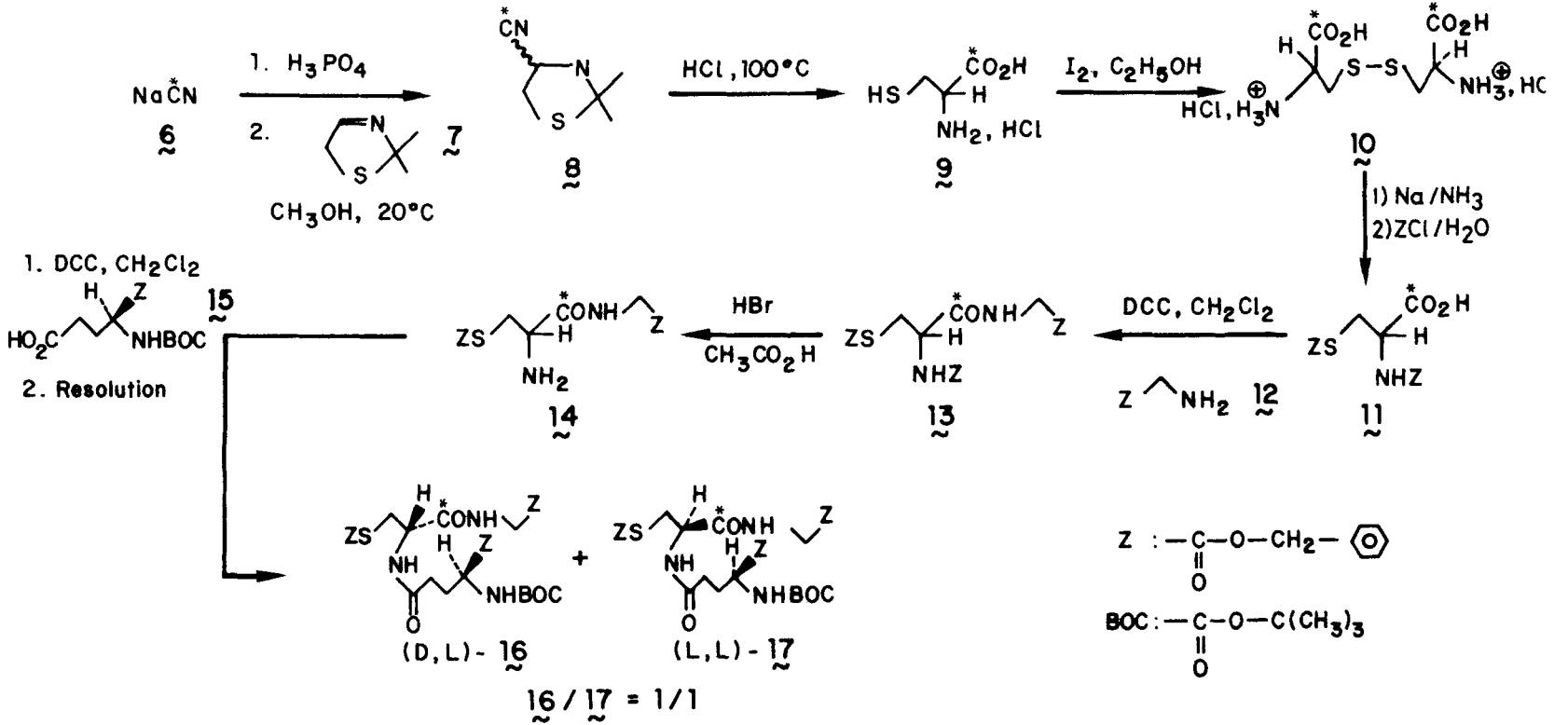
The chemical synthesis of 21, through 18 (schemes 2 and 3), requires :

- appropriate protecting groups for the peptide synthesis, especially for the thiol function of the cysteinyl residue.
- a simple and effective resolution of the diastereoisomers 16/17 of the labelled protected tripeptide, which has not yet been described, to our knowledge, for glutathione or modified analogues such as 16 and 17.

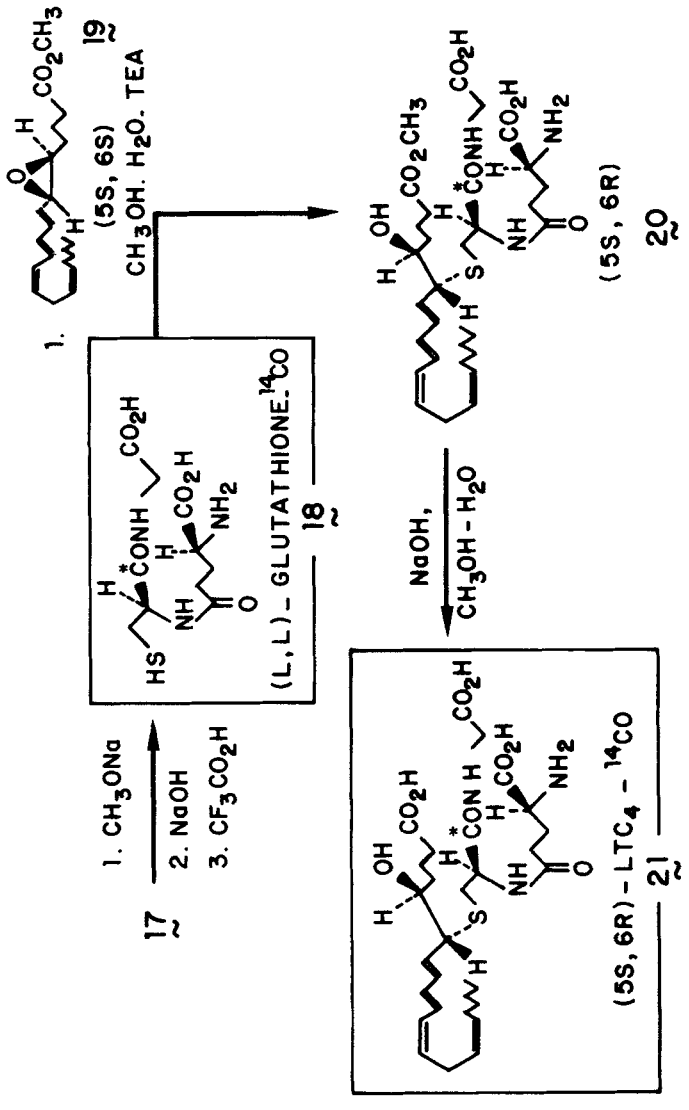
METHODS AND RESULTS

2,2-Dimethylthiazoline 7 [10] reacted with [^{14}C] hydrocyanic acid to give 2,2-dimethyl-thiazolidine [^{14}CN] cyanhydrin 8, which was hydrolyzed to D,L-[1- ^{14}C] cysteine hydrochloride 9. The latter unstable compound was oxidized in the usual way to give stable [^{14}C] cystine hydrochloride 10 (mixture of DL- and meso-forms). A thorough purification of hydrochloride 10 by liquid chromatography on a Dowex-H$^+$ ion-exchange column was necessary for the following step. Thus, hydrochloride 10 (S.A. = 100.0 mCi/mmol ; 3.7 GBq/mmol) was obtained in three steps from Na^{14}CN 6 with an overall radiochemical yield of 39 % and a radiochemical purity of 99 %.

The sodio thiol derivative of 10 was then protected by benzyloxycarbonylation to give racemic 11 with a 44 % yield. This Z protecting


NaCN
6
1. H3PO4
2.
7
CH3OH, 20°C
8
HCl, 100°C
9
I2, C2H5OH
10
1) Na/NH3
2) ZCl/H2O
11
DCC, CH2Cl2
12
13
HBr
CH3CO2H
14
1. DCC, CH2Cl2
15
2. Resolution
(D,L)- 16
(L,L)- 17
16 / 17 = 1/1
Z : —C(=O)—O—CH2—
BOC : —C(=O)—O—C(CH3)3

SCHEME 2


1.
(5S, 6S)
19
CH3OH. H2O. TEA
(L, L) - GLUTATHIONE-14CO
18
1. CH3ONa
2. NaOH
3. CF3CO2H
17
NaOH,
CH3OH - H2O
(5S, 6R)
20
(5S, 6R) - LTC4 - 14CO
21

SCHEME 3

group [12] has numerous advantages including easy UV detection at 254 nm, and good stability in the conditions used for the peptide synthesis [13] (chemoselective deprotection of $-NH_2$ and -SH functions, without racemisation of asymmetric centers).

11 was then coupled [13] with benzyl glycinate 12 to give 13 (yield 80.5 %). Chemoselective deprotection of the $-NH_2$ function of 13 gave 14. This latter unstable compound 14 was then reacted with BOC-L-Glu (α-O-Bz) 15 (FLUKA), which led to diastereoisomeric mixture of protected glutathione derivatives (D,L)- 16 and (L,L)- 17, separated by preparative liquid chromatography.

The chemical structures of both diastereoisomers 16 and 17 have been confirmed by ^{1}H-NMR and MS. Their absolute chiralities were thus unambiguously determined by comparison of their spectral properties (chemical shifts and coupling constants) and their physico-chemical properties (R_F by TLC on silica gel and t_R by HPLC with an authentic non radioactive standard of (L,L) absolute chirality, prepared from commercial L-cysteine according to the same reaction scheme). The (L,L) diastereoisomer 17 (the most polar in normal phase), after three successive deprotections, leads to (L,L)-[Cys-^{14}CO]-glutathione 18 (yield : 64 % from 17, S.A. = 50.0 mCi/mmol, radiochemical purity : 93 %).

Methyl (5S,6S)-oxido-(7E,9E,11Z,14Z)-eicosatetraenoate 19 [14] was opened [15] in position 6 by (L,L)-glutathione-^{14}C 18 in presence of 4-hydroxy-2,2,6,6-tetramethyl piperidine N-oxide as radical scavenger, to give (5S,6R)-LTC_4-^{14}C 20 (yield : 25 % after HPLC purification) which was saponified to (5S,6R)-LTC_4-^{14}C 21 (yield : 75 %, S.A. = 50.0 mCi/mmol - 1.85 GBq/mmol ; radiochemical purity : 97 %). 21 shows the triplet characteristics of the triene structural unit in UV spectroscopy. Moreover, it co-migrates in reverse-phase HPLC with an authentic sample of LTC_4 [14].

EXPERIMENTAL

UV spectra were recorded on a UVIKON 860 (KONTRON) apparatus (range 190-700 nm). ^{1}H-NMR spectra were performed on a WP 300 BRUKER apparatus operated at 300 MHz and equipped with an ASPECT 3000 calculator. Mass spectra were determined on a FINNIGAN 4610 spectrophotometer. Liquid scintillation counting of radioactive solution was carried out on a LKB WALLAC (1211 RAKBETA) apparatus. Analytical and preparative HPLC systems used a WATERS pump (model 590 or 590 EF) equipped with a UV MERCK L 3000 detector, with photodiode array and a BERTHOLD LB 504 radioactivity detector. Some purifications were effected by adsorption chromatography on a silica gel column MERCK type 60H (15 µm) and a MINIPREP LC JOBIN-YVON apparatus (pressure 10 bars). Solvent pressure may vary from 1 to 10 bars. Chemical

purity controls were performed by TLC (silica gel MERCK 60 F-254 or silanized silical gel WHATMAN KC 18 plates).

Cystine-[1-^{14}C]-hydrochloride 10

7 (31.3 mM, 3.6 g) dissolved in 3 ml of anhydrous methanol, was frozen in liquid nitrogen. [^{14}C] HCN (30.3 mM, 1589 mCi, 58.8 GBq, S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol) dried on P_2O_5 was then added under vacuo. After stirring for 17 h at -20°C, the homogeneous yellow solution containing crude 9 was frozen in liquid nitrogen. Concentrated HCl (330 mM, 30 mL) was then added dropwise under argon. The resulting solution was stirred for 3 h at 40-50°C. After cooling and concentration under partial vacuum (elimination of methanol), 18 mL of water were added to the reaction mixture, the solution was heated to 100°C for 4 h, diluted with methanol (150 mL) and 1 g active carbon added. The heterogeneous mixture was filtered off on a LS MILLIPORE filter and the residue submitted to vacuum distillation. The mixture of crude 9 + 10, after addition of methanol (50 mL) was oxidized by dropwise addition of a 0.5 M ethanolic iodine solution until persistent brown coloration. After vacuum distillation, crude 10 was purified on a ion-exchange Dowex 50 W-12 H^+ form column (200-400 meshs) eluted by 2N HCl. [1-^{14}C] cystine hydrochloride 10 (620 mCi, 22.9 GBq) was obtained in 39 % yield (S.A. = 100.0 mCi/mmol, 3.7 GBq/mmol, radiochemical purity : 99 %).

N,S-Bis-(benzyloxycarbonyl)-D,L-[1-^{14}C] cysteine 11

After elution with a 1N ammonia solution on a Dowex 50 W 12 H^+ form column, 10 base (2.25 mM, 225 mCi, 8.32 GBq, S.A. = 100.0 mCi/mmol, 3.7 GBq/mmol) obtained as white crystals was vacuum dried on P_2O_5 for 18 h, then dissolved under nitrogen in liquid ammonia (50 mL) at -40°C.

Sodium turnings were then added till a persistent blue coloration. After ammonia evaporation under N_2, to the sodium thiolate formed on 10 (white crystals) were added, under N_2 overpressure, 10 mL of boiled water degassed with argon. The aqueous solution was brought to pH 10 by 1N HCl, cooled to -15°C and benzyloxycarbonyl chloride (16.8 mM, 2.875 g) was then added dropwise, the pH being kept between 9 and 10 by 2N NaOH. After 90 m reaction at -5°C, the mixture was acidified to pH 1 by 1N HCl and then saturated by NaCl, extracted with ethyl acetate (3 x 75 ml). The organic solutions were gathered, dried over Na_2SO_4 then filtered off on a LS 5 µ MILLIPORE filter. After vacuum distillation, crude 11 was purified by liquid chromatography on a silica gel column. 11 eluted successively by CH_2Cl_2-AcOEt (50-50) and CH_2Cl_2-AcOEt-CH_3CO_2H (50-50-0.5) was obtained radiochemically pure (100 mCi, 3.7 GBq, 44 % yield, S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol).

. TLC on MERCK 60 F-254 plates : CH_2Cl_2-AcOEt-CH_3CO_2H (50-50-0.5) R_f = 0.25.

. ^{1}-H-NMR ($CDCl_3$, TMS standard) : δ = 3.27 ppm (dd, 1H, - CH_2S-, J = 6.9 Hz) ; 3.45 ppm (dd, 1H, -CH_2S-, J = 4.4 Hz) ; 4.65 ppm (m, 1H, C<u>H</u>-CO_2H) ; 5.10 ppm (s, 2H, -<u>CH_2</u>Ar) ; 5.18 ppm (s, 2H, -<u>CH_2</u>Ar) ; 7.30 ppm (s, 10H, -Ar).

. MS (EI) : m/e (%) = 298.0 (1.5) ; 283.0 (0.2) ; 257.0 (0.8) ; 211.0 (3.0) ; 107.6 (55.8) ; 91.5 (100.0).

<u>Benzyl N-[N,S-bis(benzyloxycarbonyl)-D,L-[1-^{14}C] cysteinyl]glycinate 13</u>

Dry <u>11</u> (2.05 mM, 100 mCi, 3.7 GBq, S.A. = 50.0 mCi/mmol, 185 GBq/mmol) and dicyclohexylcarbodiimide (2.4 mM, 500 mg) were stirred under nitrogen for 3 h at 0°C in presence of triethylamine (242 mg, 2.4 mM) and benzyl glycinate <u>12</u> (808 mg, 2.4 mM, tosylate salt) in 7 ml anhydrous dichloromethane. The mixture was then hydrolyzed and extracted with 5 x 15 ml CH_2Cl_2. The organic solutions, dried on $MgSO_4$, are filtered off on a LS 5 µ MILLIPORE filter. After vacuum distillation, crude <u>13</u> was purified by liquid chromatography on a silica gel column eluted by CH_2Cl_2-AcOEt (93-7). Pure <u>13</u> (80.5 mCi, 2.97 GBq) was obtained (yield 80 %, S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol, radiochemical purity : 98 %).

. TLC on MERCK 60 F-254 plates : CH_2Cl_2-AcOEt (93-7), R_f = 0.25.

. ^{1}H-NMR ($CDCl_3$, TMS standard) : δ = 3.18 ppm (dd, 1H, -CH_2S-, J = 14.6 Hz) ; 3.35 ppm (dd, 1H, -CH_2S-, J = 4.4 Hz) ; 4.05 ppm (m, 2H, -CH_2CO_2-) ; 4.47 ppm (m, 1H -CH_2<u>CH</u>(cys)) ; 5.10 ppm (s, 2H, -CH_2Ar) ; 5.15 ppm (s, 2H, -CH_2Ar) ; 5.20 ppm (s, 2H, -CH_2Ar) ; 5.65 ppm (m, 1H, NH(cys)) ; 6.75 ppm (m, 1H, NH(Gly)) ; 7.30 ppm (s, 15H, -Ar).

. MS (CI) : m/e = 554 (M + NH_4^+, 6.5 %), 537 (MH^+, 39.0 %).

<u>Benzyl N-[(benzyl (N-tertiobutoxycarbonyl-N-γ-L-glutamate)-S-benzyloxycarbonyl-[1-^{14}C] cysteinyl] glycinate diastereoisomers 16 and 17</u>

1.03 mM of <u>13</u> (50 mCi, 1.85 GBq, S.A. = 50 mCi/mmol, 1.85 GBq/mmol) were stirred at 20°C for 2 h in an anhydrous 5 % HBr acetic acid solution. The mixture was then dried, dissolved twice in a 25 ml methanol-ether (1/1) mixture and eventually evaporated again to dryness, in order to eliminate excess HBr. <u>14</u> obtained was used in the next step without further purification.

To crude <u>14</u> (50.0 mCi, 1.85 GBq) and dicyclohexylcarbodiimide (247 mg, 1.2 mM) dissolved in 5 ml anhydrous CH_2Cl_2 containing 230 µl of

triethylamine was added under N_2 at 0°C BOC-L-Glu-(α-OBz) 15 (FLUKA, 404 mg, 1.2 mM) dissolved in 5 ml anhydrous CH_2Cl_2. After 1h reaction at 0°C and treatment similar to 13, both diastereoisomers 16 and 17 were separated and purified by liquid chromatography on a silica gel column eluted by $CHCl_3$-AcOEt (90-10). 16 (9.1 mCi, 336.7 MBq, S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol) and 17 (9.6 mCi, 355.2 MBq, S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol) were obtained radiochemically pure. The 17 diastereoisomer of natural chirality, after deprotection and coupling, was obtained with a 20 % yield.

. Chromatographic results

Support	Solvents	Detection	16 (D,L)	17 (L,L)
TLC : Silica gel Merck 60 F-254 plates	$CHCl_3$-AcOEt 7-3	UV at 254 nm	R_f = 0.37	R_f = 0.31
HPLC : Analytical column Prolabo SSW	$CHCl_3$-AcOEt 9-1 Flow rate: 1 ml/m	UV at 254 nm	t_R = 12 m	t_R = 15 m

. ^{1}H-NMR ($CDCl_3$, TMS standard)

Diastereo-somers		-tBu	-NHCOCH$_2$- (Glu)	-CH$_2$S- (Cys)	-CONHCH$_2$- (Gly)	-CH< (Glu)	-CH< (Cys)	-CH$_2$Ar	-Ar
6	δ(ppm)	1.35(s)	2.10(m)	3.25(dd) 3.35(dd)	3.85(dd) 4.12(dd)	4.35(m)	4.68(m)	5.65(m)	7.30(s)
	J (Hz)			14.8 8.7	17.7 5.5				
7	δ(ppm)	1.40(s)	2.15(m)	3.22(dd) 3.35(d))	3.95(dd) 4.08(dd)	4.35(m)	4.65(m)	3.65(s)	7.30(s)
	J (Hz)			14.7 8.1	18.2 5.4				

. MS (CI, 16) : m/e (%) = 741 ($M + NH_4^+$, 31.8 %) ; 624 ($M\text{-}BOC^+$, 41.0 %).

. MS (CI, 17) : m/e (%) = 741 ($M + NH_4^+$, 9.8 %) ; 624 ($M\text{-}BOC^+$, 6,0 %).

N-[N-γ-L-glutamyl-L-[1-^{14}C] cysteinyl] glycine 18
(or [Cys-^{14}CO]-glutathione)

30.9 mM of 17 (1.5 mCi, 55.5 MBq, S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol) dissolved under argon in 1 ml anhydrous degassed methanol, were added at 20°C to a 2.3 M methanolic CH_3ONa solution (168 µM, 73 µL). After stirring for 4 m, were injected successively distilled water (300 µl) and 690 µM (300 µL) of the preceding 2.3 M methanolic CH_3ONa solution. After 2 h 30 reaction at

20°C, the mixture was cooled at 0°C, acidified under N_2 by 1N HCl to pH 2. The aqueous phase was extracted by 2 x 2 mL AcOEt. The organic phase was dried ($MgSO_4$), filtered (LS 5 µ MILLIPORE filter) and evaporated to dryness. The crude residue (1.5 mCi) was then stirred at 0°C for 30 m under N_2 in CF_3CO_2H/CH_2Cl_2 (1/1) (6 ml). After vacuum distillation, the crude radioactive (L,L)-glutathione obtained was purified by HPLC on a semi-preparative reverse phase DUPONT-ZORBAX ODS column (10 mm x 250 mm) eluted by the mixture H_2O-H_3CO_2H (100-0.1). 18 (890 µCi, 32.9 MBq) was obtained with 64% yield from 17 (S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol) and a radiochemical purity of 93 %.

. HPLC : analytical column (0.5 mm x 250 mm) of silanized silica gel DUPONT-ZORBAX ODS. H_2O-CH_3CO_2H (100-0.1). Flow rate : 1 ml/m, t_R = 4 m.

. ^{1}H-NMR (D_2O, external standard : TMS) : δ = 2.35 (q, 2H, -$COCH_2$-CH_2- (Glu), J = 7.3 Hz) ; 2.75 (dt, 2H, -$COCH_2$-CH_2-(Glu), J = 7.6 and 2.0 Hz) ; 3.15 (dd, 2H, -CH_2S-, J = 6.2 and 2.0 Hz) ; 4.08 (t, 1H, CH-(Glu), J = 6.9 Hz) ; 4.22 (s, 2H, -CH_2-(Gly)) ; 4.78 (t, 1H, CH- (Cys)).

N-[S-[1-(4-Methoxycarbonyl-1-hydroxybutyl) pentadeca-(2E,4E,6Z,9Z) tetraenyl]-N-γ-L-glutamyl-L-[1-^{14}C] cysteinyl] glycine 20
(or (5S,6R)-[Cys-^{14}CO]-LTC_4 methyl ester)

18 (15,6 µM, 4,8 mg, 756 µCi, 20,0 MBq, S.A. = 50 mCi/mmol, 1.85 GBq/mmol, radiochemical purity : 93 %) were dissolved under N_2 in 7 ml of the mixture CH_3OH-Et_3N-H_2O (82.3-3.4-14.3) in presence of 4-hydroxy-2,2,6,6-tetra-methylpiperidine N-oxide (15.6 µM, 2.6 mg). After addition of 31.3 µM (10.4 mg) of (5S,6S)-LTA_4 methyl ester 19 dissolved in 3 ml of CH_3OH-Et_3N (96-4), the reaction mixture was stirred at 20°C for 17 h. Vacuum distillation of the latter resulted in crude 20 which was purified by reverse phase HPLC on a semi-preparative DUPONT-ZORBAX ODS column (10 mm x 250 mm) eluted by CH_3OH-H_2O (75-25) buffered to pH 5.6 by ammonium acetate. Thus, 20 (189 µCi, 6.99 MBq) was obtained radiochemically pure (yield 25 %, S.A. = 50.0 mCi/mmol; 185 GBq/mmol).

. HPLC :
- silanized silica gel DUPONT-ZORBAX ODS analytical column (0.5 x 250 mm).
- elution by CH_3OH-H_2O (75-25) buffered to pH 5.6 by ammonium acetate. Flow rate : 1 ml/m.
  t_R = 10 m.

. UV (CH_3OG) : λ_{max} = 272.0 ; 281.0 ; 292.0 nm.

N-[S-[1-(4-carboxy-1-hydroxybutyl) pentadeca-(2E,4E,6Z,9Z)-tetraenyl] -N- λ-L-glutamyl-L-[1- ^{14}C] cysteinylç glycine 21
(or (5S,6S)-[Cys-^{14}CO] -LTC_4)

20 (2.9 µM, 141 µCi, 5.2 MBq, S.A. = 50.0 mCi/mmol, 1.85 GBq/mmol) dissolved in 1 ml distilled water was stirred at 4°C under argon with 2 ml of a 0.15 M NaOH solution in the mixture CH_3OH-H_2O (75-25) (300 µM NaOH). After 1 h 30 m reaction at 4°C the solution was neutralized by CH_3CO_2H (170 µl) and then vacuum distilled to give crude 21, which was purified by reverse phase HPLC on a semi-preparative DUPONT-ZORBAX ODS column (10 mm x 250 mm) eluted by CH_3OH-H_2O (75-25) buffered at pH 5.6 by ammonium acetate.
21 (105 µCi, 3.88 MBq, S.A. = 50 mCi/mmol, 1.85 GBq/mmol, yield : 74 %) was obtained radiochemically pure and had to be stored at -80°C sheltered from light and under argon in the purification solvent at pH 8.5 (addition of a 12 M ammonia solution).

. HPLC :
- Silanized silica gel DUPONT-ZORBAX ODS analytical column (0.5 mm x 250 mm)
- Elution by CH_3OH-H_2O buffered at pH 5.6 by ammonium acetate.
  Flow rate : 1 ml/m.
  t_R = 4 m.

. UV (CH_3OH) : λ_{max} = 271.0 ; 280.6 ; 291.6 nm.

CONCLUSION

Carbon-14 labelled (L,L)-glutathione 18 and (5S,6R)-LTC$_4$ 21 were prepared by chemical synthesis. Both compounds are radiochemically pure and have a specific activity of 50 mCi/mmol (1.85 GBq/mM). The biological use of ^{14}C-LTC$_4$ is being investigated.

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