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(54) Title: A PROCESS FOR THE PREPARATION OF GLUTATHIONE S-ACYL DERIVATIVES, COMPOUNDS OBTAINED FROM SAID PROCESS AND AN INTERMEDIATE USEFUL FOR THE PREPARATION THEREOF

(57) Abstract

A selective and high yield process for S-acylating glutathione, comprising the reaction between glutathione and an acyl chloride or a carboxylic acid anhydride in trifluoroacetic acid is described.

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A PROCESS FOR THE PREPARATION OF GLUTATHIONE S-ACYL DERIVATIVES, COMPOUNDS OBTAINED FROM SAID PROCESS AND AN INTERMEDIATE USEFUL FOR THE PREPARATION THEREOF

The present invention relates to a process for the preparation of glutathione S-acyl derivatives and esters thereof, to novel derivatives obtained from said process. The invention also relates to pharmaceutical compositions containing said glutathione S-acyl derivatives as active ingredients.

In these last years glutathione (GSH) has given rise to an increasing interest for the potential therapeutic effects as well as for metabolism thereof and for derivatives therefrom. GSH N,S-acyl derivatives were described, for instance, in Japanese patent applications (see Chemical Abstracts 97-7222755S and 77-5493M.

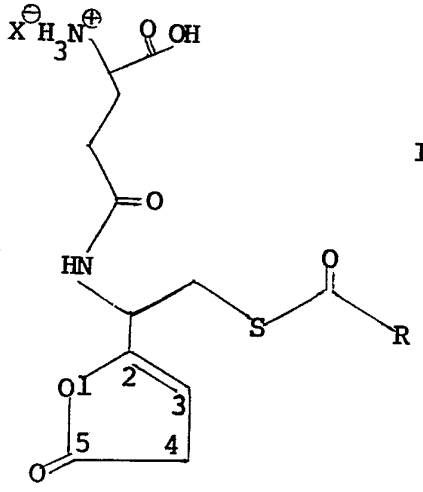
Moreover, glutathione S-acyl derivatives, whose sensitivity to enzymatic hydrolysis by specific human liver-esterases was studied (Biochemistry, Vol. 12, No. 20, 1973, 3938-3943), have already been described. Glutathione S-acyl derivatives are stable in serum and glutathione itself is released by the action of specific liver esterases; this makes possible using said derivatives as glutathione "pro-drug".

The hitherto known methods for the selective acylation of glutathione thio group, without involving the free amino group, are suitable for small scale preparations such as laboratory ones, but not for large industrial applications. For instance, S-acetyl and S-butyryl-glutathione have been prepared by reacting glutathione with thioacetic and thiobutyric acids in 40

and 20% yields, respectively (J. Biol. Chem. 1954, 206: 327-333). Also enzymatic methods are known to prepare glutathione S-acyl derivatives (J. Biol. Chem. 1951, 190: 685). The above mentioned enzymatic methods are clearly useful only for laboratory-scale preparations.

It has now been found a method for the specific preparation of glutathione S-acyl derivatives in high yields, starting from low cost and easily available reagents such as acyl halides or acid anhydrides.

Particularly, the process of the invention consists in reacting glutathione or esters thereof with acyl halides or acid anhydrides in trifluoroacetic acid. The stoichiometric ratios are not particularly critical but at least two acyl halide equivalents per glutathione equivalent are preferably used. Under such conditions, the amino group is completely protected against protonation and the only nucleophilic group which can react with the acylating agent is the free thiol. When two equivalents of acylating agent are used, one equivalent acylates the -SH group according to the faster reaction, while the other one induces intramolecular dehydration of the glycine moiety and 4-(H-5oxo-1,3-oxazoline derivative of formula II is accordingly formed.



II

Such an intermediate of formula II shows a keto-enol equilibrium concerning positions 4 and 5.

The obtained S-acyl-glutathiones can be esterified according to conventional methods.

Some S-acyl-derivatives, which are prepared according to the invention are new, therefore they are a further object of the invention. Such derivatives can be used in human therapy, in the form of suitable pharmaceutical formulations, for the same already well-known applications of natural GSH. The only condition required is that the acyl residue can give rise in vivo to a non toxic, physiologically compatible carboxylic acid. Examples of such acids (deriving from the corresponding acyl residues) include acetic, propionic, pivalic, phenylacetic, benzoic, 2- or 3-thenoic acids.

The compounds of the invention can be formulated in pharmaceutical compositions suitable for oral or parenteral administrations, by using conventional techniques and excipients.

The following examples further illustrate the invention.

EXAMPLE 1

While stirring under nitrogen blanquet pivaloyl chloride (14.7 g) is dropwise added to a solution of L-glutathione reduced (18.4 g) in trifluoroacetic acid (200 ml) at room temperature.

The reaction mixture is heated to 40°C and after about 20 minutes is treated with water (5 ml) and further heated at 40°C for about 20 minutes. Then the solvent is removed under reduced pressure (40°C 250 mmHg; 90°C 20 mmHg) and the resulting oil is quenched

with ethyl acetate (200 ml) leading to a white solid overnight. The crude precipitate is dissolved in warm (40°C) acetone/water (5/1, 200 ml). After cooling in an ice-water bath for about two hours the white crystalline material formed is collected, washed with the solvent (30 ml) and dried over $CaCl_2$ under vacuum (40°C, 20 mmHg, 4h) yielding γ-L-glutamyl-S-pivaloyl-L-cysteinyl-glycine (18,7 g, yield = 80%).

(i) m.p. = 201 ÷ 203°C

(ii) $[\alpha]_D^{25} = -18$ $[\alpha]_{546}^{25} = -21.6$ (c=1 in H_2O)

(iii) TLC: SiO_2 $60F_{254}$, 0.25 thickness

eluent n-propanol:acetic acid:water = 10:1:5 (v/v)

eluition time: 45'

developing reagent: 0.5% ninhydrin is hereinafter referred to as NiN

Rf= 0.53 (brown-red spot)

(iv) NMR Bruker 200 MHz: DMSO-d_6; δ(ppm), J(Hz)

^1H

1.15 (9H, S)	t-Bu
1.90 (2H, m)	Glu (β)
2.30 (2H, m)	Glu (γ)
2.95 (1H, dd, J_1=15 J_2=10)	Cys (β)
3.30 (1H, dd, J_1=15 J_2=5)	Cys (β)
3.45 (1H, t, J=7)	Glu (α)
3.70 (2H, d, J=5)	Gly (α)
4.40 (1H, m)	Cys (α)
8.42 (1H, d, J=7.5)	Cys (NH)
	(exchangeable with D_2O)
8.50 (1H, t, J=5)	Gly (NH)
	(exchangeable with D_2O)

(v) Elemental analysis (for $C_{15}H_{25}N_3O_7S$)

	C	H	N	S
Calculated (%)	46.02	6.44	10.73	8.19
Found (%)	45.9	6.47	10.7	8.11

EXAMPLE 2

Using the procedure described in the Example 1 but with modified work-up, γ-L-glutamyl-S-pivaloyl-L-cystenyl glycine hydrochloride dihydrate can be obtained.

By reacting L-glutathione reduced (15.4 g) with pivaloyl chloride as previously outlined, the residual oil obtained from the reaction mixture concentrated under vacuum is dissolved in acetic acid (100 ml) under nitrogen. At room temperature hydrogen chloride in anhydrous diethyl ether (4.7 N; 32 ml) is dropwise added to the resulting solution.

Under vigorous stirring more anhydrous diethyl ether (250 ml) is added to facilitate the crystallization of a white solid which is allowed to stand overnight under stirring. The precipitate is collected under nitrogen, washed with diethyl ether (50 ml) and dried over P_2O_5 (25°C 10 mmHg 4 h). This leads to γ-L-glutamyl-S-pivaloyl-L-cysteinyl-glycine hydrochloride dihydrate (19 g; yield = 89%)

(i) m.p. = 90 ÷ 100°C

(ii) $[\alpha]_D^{25} = -12$ $[\alpha]_{546}^{25} = -14.3$ (c=1 MeOH)

(iii) Elemental analysis (as $C_{15}H_{25}N_3O_7S.HCl.H_2O$)

	C	H	N	S	Cl
Calculated (%)	40.4	6.3	9.42	7.19	7.95
Found (%)	40.3	6,3	9.37	7.3	7.54

(iv) NMR Bruker 200MHz; DMSO-d_6: δ(ppm); J (Hz)

^1H	^{13}C	
1.15 (9H, s)	27	T-Bu
2 (2H, m)	26	Glu (ß)
2.30 (2H, m)	30	Glu (γ)
2.9 (1H, dd, J_1 = 15, J_2= 10)	30	Cys (ß)
3.25 (1H, dd, J_1 = 15, J_2 = 5)		Cys (ß)
3.7 (2H, m)	40	Gly (α)
3.85 (1H, m)	51	Glu (α)
4.4 (1H, m)	51	Cys (α)
8.35 (1H, m)		Gly (NH)
	(exchangeable with D_2O)	
8.4 (1H,d)		Cys (NH)
	(exchangeable with D_2O)	

NMR Bruker 200 MHz, D_2O

^1H	
1.25 (9H, S)	T-Bu
2.2 (2H pseudo q, J=7)	Glu (ß)
2.56 (2H, t, (J=7)	Glu ( )
3.22 (1H, dd, J_1 = 14, J_2= 8)	Cys (ß)
3.45 (1H, dd, J_1 = 14, J_2 = 5)	Cys (ß)
4.00 (1H, t, J=7)	Glu ( )
4.02 (2H, s)	Gly ( )
4.65 (1H, dd, J_1=5, J_2=8)	Cys ( )

EXAMPLE 3

Using in the procedure of Example 1 acetyl chloride, in place of pivaloyl chloride, L-glutathione reduced (15.4 g) provides, after usual work-up, a crude white solid which is dissolved in warm acetone/water (2/1; 40°C; 300 ml). The obtained solution is further diluted with more acetone (100 ml) and cooled on ice-water bath. After about two hours the white crystalline

precipitate is collected, washed with the solvent (40 ml) and dried under vacuum affording γ-L-glutamyl-S-acetyl-L-cysteinyl-glycine (14.8 g yield=85%)

(i) m.p. = 202 ÷ 204°C

(ii) $[\alpha]_D^{25}$ = -27.5 $[\alpha]_{546}^{25}$ = -32.6 (c=1.1 H_2O)

(iii) TLC: SiO_2 $60F_{254}$; 0.25 Thickness

eluent n-propanol: acetic acid: water = 10:1:5 (V/V)

eluition time: 45'

developing reagent: NiN

Rf = 0.37 (brown-red spot)

(iv) NMR Bruker 200 mHz; DMSO-d_6; S(ppm) J(Hz)

^1H

1.95	(2H, S)	Glu (ß)
2.3	(5H s + m)	Glu (γ) + $C\underline{H}_3$
2.95	(1H, dd, J_1 = 15, J_2= 10)	Cys (ß)
3.32	(1H, dd, J_1 = 15, J_2 = 5)	Cys (ß)
3.45	(1H, t, J=5)	Glu (α)
3.7	(2H, d, J=5)	Gly (α)
4.4	(1H, m)	Cys (α)
8.5	(2H, d, J=5)	Cys (NH)-Gly(NH)

(v) Elemental analysis (as $C_{12}H_{19}N_3O_7S$)

	C	H	N	S
Calculated (%)	41.25	5.48	12.03	9.18
Found (%)	41.29	5.51	12.05	9.09

EXAMPLE 4

Using in the procedure of Example 1 benzoyl chloride in place of pivaloyl chloride, L-glutathione reduced (15.4 g) affords a crude white solid which is suspended in boiling acetone/water (3/1; 400 ml) for thirty minutes. After cooling at room temperature the

white precipitate is collected by filtration and washed with acetone (20 ml). Under vacuum drying yields γ-L-glutamyl-S-benzoyl-L-cysteinyl-glycine (16 g yield=78%).

(i) m.p. = 213 ÷ 215°C

(ii) $[\alpha]_D^{25}$ = -12 $[\alpha]_{546}^{25}$ =-15 (c=0.67 DMSO)

(iii) Elemental analysis (as $C_{17}H_{21}N_3O_7S$)

	C	H	N	S
Calculated (%)	49.63	5.14	10.21	7.79
Found (%)	49.67	5.05	10.1	7.60

(iv) TLC SiO_2 $60F_{254}$ 0.25 Thickness

eluent: n-propyl OH: AcOH: H_2O= 10:1:5 (V/V)

eluition time= 45'

developing reagent= NiN

Rf= 0.52 (pink spot)

(v) NMR Bruker 200 MH_z, DMSO-d_6, δ(ppm) J(Hz)

^1H

1.9 (2H, m)	Glu (β)
2.32 (2H, m)	Glu (γ)
3.2 (1H, dd, J_1 = 14, J_2= 10)	Cys (β)
3.4 (1H, t, J = 7.5)	Glu (α)
3.6 (1H, dd, J_1 = 14, J_2=5)	Cys (β)
3.75 (2H, d, J=5)	Gly (α)
4.55 (1H, m)	Cys (α)
7.55 (2H, t)	Aro-m,m'
7.7 (1H, t)	Aro-p
7.9 (2H, d)	Aro-o,o'
8.55 (1H, d J=8)	Cys (NH)
8.75 (1H, t J=5)	Gly (NH)

EXAMPLE 5

Under nitrogen atmosphere, pivaloyl chloride (15.9

g) is dropwise added to a stirred solution of ethyl γ-L-glutamyl-L-cysteinyl-glycinate (10.9) in trifluoro-acetic acid (220 ml) at room temperature. The reaction mixture is warmed to 40°C and about fifteen minutes later is treated with water (2.5 ml) and then allowed to stand at 40°C for further fifteen minutes.

The solvent is removed under vacuum (40°C 250 mmHg; 90°C 20 mmHg) and the oily residue is treated with ethyl ether/ethyl acetate (1/1 200 ml). The overnight stirring at room temperature gives a white precipitate which is filtered off, washed with the above solvent mixture (50 ml) and dried over $CaCl_2$ in vacuo (40°C 20 mmHg 3h).

This yields ethyl γ-L-glutamyl-S-pivaloyl-L-cysteinyl-glycinate (9.2 g yield=67.5%)

(i) m.p. = 143 ÷ 145°C

(ii) IR (KBr cm^{-1}) 1740, 1635, 1520, 1460, 1210

(iii) TLC SiO_2 $60F_{254}$ 0.25 Thickness

eluent n-propanol: AcOH: H_2O=10:1:5 (V/V)

elution time: 45'

developing reagent: NiN

Rf = 0.6 (brown spot)

(iv) NMR Bruker 200 MH_z, DMSO-d_6; δ (ppm); J(Hz)

^1H

1.12 (9H, s)	t-Bu
1.15 (3H, t, J=7.5)	$CH_2\underline{CH}_3$
1,95 (2H, m)	Glu (β)
2.30 (2H, m)	Glu (γ)
2.95 (1H, dd)	Cys (β)
3.20 (1H, dd,)	Cys (β)
3.68 (1H, t)	Glu (α)

3.75 (2H, m) Gly (α)

4.05 (2H, q, J=7.5) $C\underline{H}_2$-CH_3

4.40 (1H, m) Cys (α)

8.30 (1H, d) Cys (NH)

8.55 (1H, t) Gly (NH)

(v) $[\alpha]_D^{25}$ = -24.9 $[\alpha]_{546}^{25}$ = -29.1 (c=1 MeOH)

EXAMPLE 6

Using in the procedure of Example 5 benzoyl chloride in place of pivaloyl chloride, ethyl γ-L-glutamyl-S-benzoyl-L-cysteinyl glycinate is obtained (70% yield).

(i) m.p. = 157 ÷ 159°C

(ii) $[\alpha]_D^{25}$ -25 $[\alpha]_{546}^{25}$ -29 (c=1; H_2O)

(iii) TLC SiO_2 $60F_{254}$ 0.25 Thickness

eluent: n-P_2OH: AcOH: H_2O = 10:1:5

elution time: 45'

developing reagent: NiN

Rf= 0.61 (brown spot)

(iv) NHR bruker 200 MHz DMSO-d_6 δ(ppm) J(Hz)

^1H

1.15 (3H, t, J=8) $CH_2C\underline{H}_3$

2.00 (2H, m) Glu (β)

2.35 (2H, m) Glu (γ)

3.2 (1H, dd) Cys (β)

3.55 (1H, dd) Cys (β)

3.65 (1H, m) Glu (α)

3.8 (2H, d) Gly (α)

4.05 (2H, d, J=8) $C\underline{H}_2$-CH_3

4.55 (1H, m) Cys (α)

7.50-7.9 (5H, t+t+d) H-Aro

8.4 (1H, d) Cys (H)

8.55 (1H, t) Gly (NH)

EXAMPLE 7

Using in the procedure of example 5 acetyl chloride in place of pivaloyl chloride, ethyl γ-L-glutamyl-S-acetyl-L-cysteinyl glycinate is obtained (86.9% yield).

(i) m.p. = 130 ÷ 132°C

(ii) $[\alpha]_D^{25}$ -24.5 $[\alpha]_{546}^{25}$ -28 (c=1.03 in H_2O)

(iii) TLC SiO_2 $60F_{254}$ 0.25 Thickness

eluent: nP_2OH; AcOH; H_2O = 10:1:5 (v/v)

elution time: 45'

developing reagent: 0.5% NiN

Rf = 0.55 (brown-red spot)

(iv) NMR Bruker 200 MHz DMSO-d_6 δ (ppm) J (Hz)

^1H

1.2	(3H, t, J=6)	$-CH_2\underline{CH_3}$
1.9	(2H, m)	Glu (β)
2.3	(5H, s + m)	Glu (γ) + $\underline{CH_3}$-H
2.95	(1H, d, d)	Cys (β)
3.30	(1H, d, d)	Cys (β)
3.70	(1H, t)	Glu (α)
3.80	(2H, d)	Gly (α)
4.05	(2H, q, J=6)	$-\underline{CH_2}CH_3$
4.40	(1H, m)	Cys (α)
8.30	(1H, d)	Cys (-NH)
8.55	(1H, t)	Gly (-NH)

EXAMPLE 8

Following the procedure described in Example 3 but using two molar equivalents of acetic anhydride, instead of acetyl chloride, the same product γ-L-glutamyl-S-acetyl-L-cysteinyl-glycine is obtained.

(67%) yield).

EXAMPLE 9

By using the same procedure described in Example 1 but reacting reduced glutathione (12.5 g) with an 1.2 molar excess of pivaloyl chloride (5.9 g) pure γ-L-glutamyl-S-pivaloyl-L-cysteinyl-glycine (11.3 g) is obtained. (71% yield)

EXAMPLE 10

Using in the procedure of example 1 phenylacetyl-chloride in place of pivaloyl-chloride, γ-L-glutamyl-S-phenylacetyl-L-cysteinyl-glycine is obtained (80% yield).

(i) m.p. = 195 ÷ 197°C

(ii) $[\alpha]_D^{25}$ = -30.5 $[\alpha]_{546}^{D}$ = -36 (C=1.05 - DMSO)

(iii) TLC SiO_2 $60F_{254}$ 0.25 Thickness

eluent : n-P_2OH; AcOH; H_2O = 10:1:5 (v/v)

elution time: 45'

developing reagent: 0.5% NiN

Rf = 0.64 (brown-red spot)

(iv) NMR Bruker 200 MHz DMSOd_6 δ(ppm) J(Hz)

^1H

1.90 (2H, m)	Glu (β)
2.30 (2H, m)	Glu (γ)
2.95 (1H, d, d)	Cys (β)
3.30 (1H, d, d)	Cys (β)
3.45 (1H, t)	Glu (α)
3.20 (2H, d)	Gly (α)
3.10 (2H, s)	CH_2-C_6H_5
4.40 (1H, m)	Cys (α)
7.27 (5H, m)	C_6H_5
8.45 (1H, d)	Cys (-NH)

8.55 (1H, t) Gly (NH)

Elemental analysis (as $C_{18}H_{23}N_3O_7S$)

	C	H	N	S
Calculated (%)	50.81	5.45	9.87	7.53
Found (%)	50.57	5.45	9.91	7.46

TABLE

The enzymatic hydrolysis specific activity of thioester bond is expressed as -SH released nmoles/min/mg of proteins. Tests with different concentrations of proteins and with different concentrations of substrate proved the enzymatic nature of the hydrolysis. Spontaneous hydrolysis of the substrates ranges from 1% to 10% maximum. The values are expressed as the mean ± SD on 6 tests.

GSH derivative (0,16 mM)	Cytosol	Serum	Ratio
S-acetyl	10,8 ± 1,5	0	00
S-pivaloyl	6,9 ± 0,7	0	00
S-phenylacetyl	6,7 ± 0,9	0,05 ± 0,003	134,0
S-benzoyl	3,2 ± 1,1	0,04 ± 0,005	80,0
S-thenoyl	4,1 ± 1,0	0,06 ± 0,001	63,0
S-benzoyl-diethyl-ester	1,45 ± 0,8	0,07 ± 0,005	18,5
S-benzoyl-monoethyl-ester	0,6 ± 0,08	0,10 ± 0,004	6,0

As far as the ratio of intracellular to extracellular hydrolysis rate is concerned, the following scale can be established: S-acetyl > S-pivaloyl > S-phenylacetyl > S-benzoyl > S-thenoyl > S-benzoyl-diethylester > S-benzoyl-monoethylester.

CLAIMS

1. A process for the preparation of glutathione S-acyl derivatives, characterized in that glutathione or esters thereof are reacted with carboxylic acid anhydrides or with acyl halides in the presence of trifluoroacetic acid.

2. A process according to claim 1, characterized in that the ratio of acyl chloride equivalents to glutathione equivalent is least 2:1.

3. A process according to claim 2, wherein the acyl chloride is selected from acetyl, pivaloyl, phenacetyl, benzoyl, 2- or 3-thenoyl chlorides.

4. A process according to anyone of the previous claims, characterized in that the acylating reaction is followed by hydrolysis with water to give S-acyl-glutathiones or esters thereof.

5. S-acyl glutathione derivatives obtainable by the process as claimed in claims 1 - 4.

6. An S-acyl-glutathione selected from:
S-pivaloyl-glutathione, S-phenylacetyl-glutathione, S-benzoyl-glutathione and S-thenoyl-glutathione.

7. Pharmaceutical compositions containing an S-acyl-glutathione of claim 6 as active ingredient.

INTERNATIONAL SEARCH REPORT

International Appli n No PCT/EP 91/01154

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 5/02 A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOCHEMISTRY, vol. 12, no. 20, 25 September 1973, American Chemical Society, L. UOTILA: "Preparation and assay of glutathione thiol esters. Survey of human liver glutathione thiol esterases", pages 3938-3943, see the compounds of table 1, especially compound no. 8 (cited in the application) ---	5
X	THE JOURNAL OF ANTIBIOTICS, June 1990, (Tokyo, JP), M.A. FERRERO et al.: "V. Biosynthesis of benzylpenicillin (G), phenoxymethylpenicillin (V) and octanoylpenicillin (K) from glutathione S-derivatives", pages 684-691, see the whole article --- -/-	5-6

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24-09-1991	08 OCT 1991
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	THE JOURNAL OF ORGANIC CHEMISTRY, vol. 47, no. 7, 26 March 1982, American Chemical Society, A.P. HAGEN et al.: "Reaction of trifluoroacetic acid with alcohols, phenols, ethers, and their sulfur analogues", pages 1345-1347, see the whole article	1-4