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SYNTHESIS AND CHARACTERIZATION OF ISOTOPICALLY-LABELLED CYSTEINE- AND GLUTATHIONE CONJUGATES OF METHYLISOCYANATE

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SUMMARY

Four isotopically labelled analogs of *S*-(*N*-methylcarbamoyl)glutathione (SMG), the glutathione conjugate of methylisocyanate, and three isotopically-labelled derivatives of *S*-(*N*-methylcarbamoyl)cysteine (SMC), the corresponding cysteine adduct, have been prepared by reaction of glutathione and cysteine, respectively, with [1-^{13}C]-, [3-^{13}C]-, [3-^{14}C]- or [3,3,3-$^{2}H_3$]methylisocyanate. The latter species, which served as key intermediates in the syntheses, were obtained either by reaction of [1-^{13}C]acetylchloride with sodium azide (to give [1-^{13}C]methylisocyanate), or by reaction of silver cyanate with iodomethane labelled with ^{13}C, ^{14}C or deuterium (to give the methyl-substituted compounds). Condensation of labelled methylisocyanate with glutathione or cysteine proceeded smoothly and in high yield in aqueous acetonitrile solution, and afforded the target conjugates in high isotopic purity. The structures of the products were confirmed by fast atom bombardment tandem mass spectrometry (FAB/MS/MS) and by ^{1}H- and ^{13}C-NMR spectroscopy.

Key Words: [^{14}C]Methylisocyanate, [^{13}C]methylisocyanate, [^{2}H]methylisocyanate, *S*-(*N*-methylcarbamoyl)glutathione, *S*-(*N*-methylcarbamoyl)-cysteine, fast atom bombardment tandem mass spectrometry.

INTRODUCTION

S-(*N*-methylcarbamoyl)glutathione (SMG; *3*; Figure 1) is a metabolite of the investigational antitumor agent *N*-methylformamide (NMF; *1*; Figure 1) in rodents and in suspensions of isolated rat hepatocytes[1,2]. The corresponding mercapturic acid, *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine, has been identified as a

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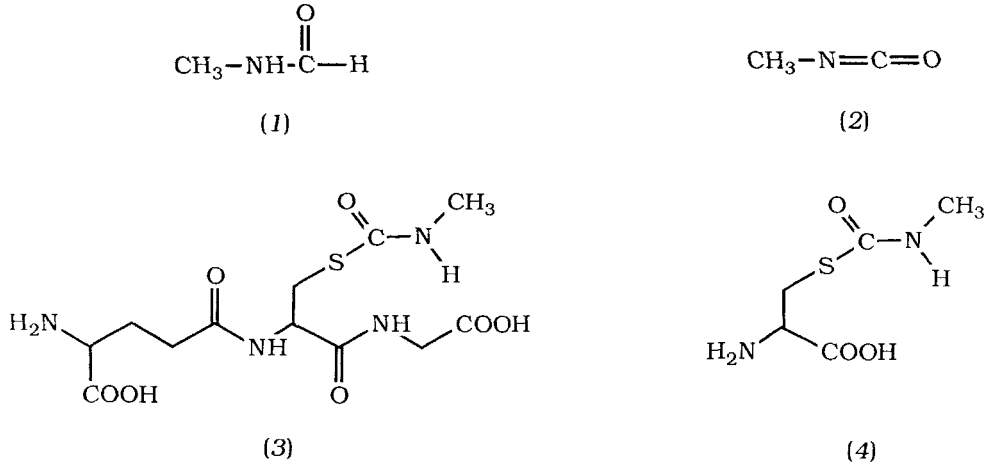
metabolite of NMF in mice and man[3], and is also found in the urine of mice and humans exposed to the industrial solvent *N,N*-dimethylformamide.[4] The mechanism by which SMG is produced during metabolism of these formamides is unknown, but its formation has been suggested to reflect the intermediacy of methylisocyanate (*2*; Figure 1), generated by net two-electron oxidation of NMF and captured subsequently by nucleophilic addition of free glutathione (GSH).[1] Recently, it has been reported that the glutathione conjugates of allyl- and benzylisothiocyanate, which are related structurally to SMG but which possess a thiocarbamate linkage between xenobiotic and GSH, revert to the parent isothiocyanates spontaneously in aqueous media.[5] Indeed, this property of these conjugates may be responsible for their observed cytotoxicity in cultures of RL-4 rat hepatocytes.[5] Since NMF is known to be hepatotoxic in rodents and humans,[6,7] it follows that methylisocyanate, produced either directly by metabolic oxidation of NMF or, possibly, indirectly through decomposition of SMG, may play a role in mediating NMF-induced hepatic injury.

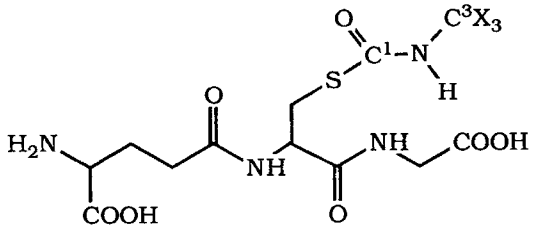
Recently, we have demonstrated the value of stable-isotope-labelled analogs of NMF in mass spectrometric and NMR studies of the biotransformation of NMF.[1,8] In order to now evaluate the role of SMG in the expression of the anti-neoplastic and hepatotoxic properties of NMF, analogs of SMG and the corresponding cysteine conjugate, *S*-(*N*-methylcarbamoyl)-cysteine (SMC; *4*; Figure 1), labelled at specific sites with stable isotopes, were required as metabolic probes.



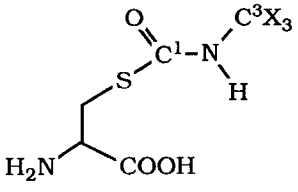
(*1*) (*2*) (*3*) (*4*)

Figure 1. Structures of NMF (*1*), methylisocyanate (*2*), SMG (*3*) and SMC (*4*).

In this paper, we describe the synthesis of SMG and SMC enriched with either ^{13}C or $[^2H_3]$ in the methyl group, or with ^{13}C at the formyl carbon. In addition, we report on the synthesis of a radiolabelled conjugate, [methyl-^{14}C]SMG, which was required as a tracer to facilitate studies of the metabolic fate of SMG in biological systems. The key intermediates in the preparation of the target compounds were appropriately labelled analogs of methylisocyanate which, in turn, were generated conveniently from either [1-^{13}C]acetylchloride or from [^{13}C]- or $[^2H_3]$iodomethane.



(*5*) $C^1=^{12}C$; $C^3=^{13}C$; $X=^1H$.
(*6*) $C^1=C^3=^{12}C$; $X=^2H$.
(*7*) $C^1=^{13}C$; $C^3=^{12}C$; $X=^1H$.
(*8*) $C^1=^{12}C$; $C^3=^{14}C$; $X=^1H$.



(*9*) $C^1=C^3=^{12}C$; $X=^2H$.
(*10*) $C^1=^{12}C$; $C^3=^{13}C$; $X=^1H$.
(*11*) $C^1=^{13}C$; $C^3=^{12}C$; $X=^1H$.

Figure 2. Structures of isotopically-enriched analogs of *S*-(*N*-methylcarbamoyl)-glutathione (*5*)-(*8*) and *S*-(*N*-methylcarbamoyl)cysteine (*9*)-(*11*).

RESULTS AND DISCUSSION

The synthesis of *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine has been accomplished previously by treatment of *N*-acetylcysteine with methylisocyanate in anhydrous pyridine,[3] while the preparation of *S*-(*N*-ethylcarbamoyl)cysteine by reaction of *N*-ethylisocyanate with cysteine in DMF has also been reported.[9] However, the poor solubility of GSH in pyridine, combined with the propensity of methylisocyanate to form substituted ureas in the presence of unprotected amines,[10] complicates the synthesis of the glutathione conjugate of methylisocyanate in organic solvents under basic conditions. In principle, this difficulty

can be circumvented by the use of suitable protecting groups for the free amino and carboxylic acid functionalities of GSH, which both improves the solubility of this tripeptide in organic solvents, and directs the reaction with methylisocyanate to the cysteinyl-thiol. The drawback of such a synthetic strategy, at least for the purposes of the present work, is that removal of the protecting groups from the synthetic product entails several steps beyond the point at which the isotopic label is incorporated.

In preliminary studies aimed at developing more suitable procedures for the synthesis of sulfur conjugates of methylisocyanate, we found that GSH, dissolved in a minimum volume of 70% acetonitrile in water, reacts smoothly (and in the absence of base) with a 2-fold molar excess of methylisocyanate in acetone to afford SMG in 90% yield. Based on this finding, we proceeded to develop syntheses of analogs of methylisocyanate labelled specifically with radioactive or stable isotopes at either the formyl (C-1) or methyl (C-3) position. The resulting methylisocyanate derivatives were then reacted with GSH or cysteine to give the desired products.

The first synthetic procedure, which led to methylisocyanate labelled at C-1, took advantage of the Curtius rearrangement of acylazides which, in turn, are accessible by reaction of acylchlorides with sodium azide.[11,12] Thus, [1-^{13}C]methylisocyanate was obtained by refluxing [1-^{13}C]acetylchloride with sodium azide in toluene, and the product was allowed to react, as outlined above, with GSH or cysteine to give the labelled conjugates (*7*) and (*11*), respectively (Figure 2). Despite claims of improved yields of methylisocyanate in the above process by the addition of tetrabutyl ammonium bromide as a phase transfer catalyst,[12,13] no appreciable benefit of this agent was noted in our hands. The ^{13}C-enriched glutathione conjugate (*7*) obtained by this route afforded a ^{1}H-NMR spectrum closely similar to that of GSH itself, although it exhibited the expected *N*-CH_3 resonance at 2.78 ppm and the ABX pattern (centered at 3.2 and 4.6 ppm) for the cysteinyl α-CH and β-CH_2 resonances, characteristic of *S*-substituted derivatives of GSH. (The magnetic inequivalence of the latter β-methylene protons arises from their diastereotopic relationship, coupled with the fact that rotation along the axis of the cysteinyl α-β bond is restricted by substitution of the thiol

functionality). Further verification of the structure of (*7*) was obtained by ^{13}C-NMR, which afforded a spectrum dominated by a singlet at 172 ppm, assigned to the labelled carbonyl carbon, and by fast atom bombardment mass spectrometry (FAB/MS), which provided an abundant MH$^+$ ion (at *m/z* 366). The corresponding ^{13}C-labelled cysteine conjugate (*11*) was characterized in a similar fashion by ^{1}H- and ^{13}C-NMR and by FAB/MS.

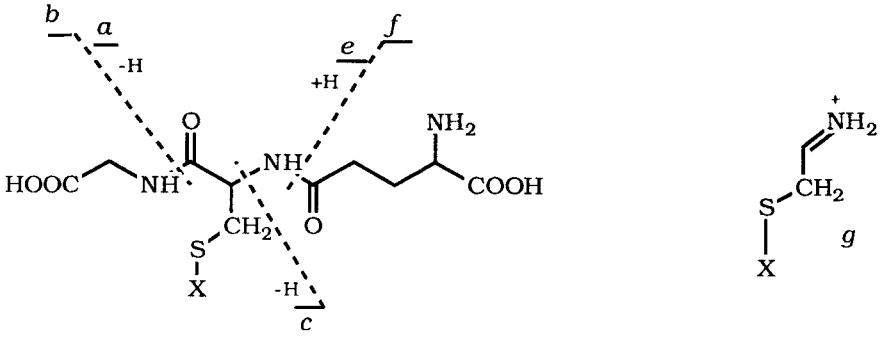
In order to prepare conjugates of methylisocyanate labelled at the methyl (C-3) position, a different synthetic approach was required. The reaction between alkyl halides and metal cyanates is a well-known pathway to alkyl isocyanates,[14] proceeding either by alkylation of the nitrogen of the mesomeric cyanate anion by the alkyl halide, or by rearrangement of the alkyl cyanate to the thermodynamically more stable alkyl isocyanate isomer.[15] When the alkyl halide in question is, e.g. iodomethane (of which a variety of labelled derivatives are available from commercial sources), this reaction provides an attractive route to derivatives of methylisocyanate labelled selectively at the C-3 position. Thus, [^{13}C]-, [$^2H_3$]- or [^{14}C]iodomethane was allowed to react at room temperature with silver cyanate in acetonitrile for 5 days to afford the corresponding methyl-labelled analogs of methylisocyanate, which were isolated by microdistillation and employed, as before, as precursors of the target sulfur conjugates. The latter were obtained in moderate yields by this route, and were characterized readily by their NMR and FAB mass spectra. The ^{13}C-labelled glutathione conjugate (*5*), for example, afforded a ^{1}H-NMR spectrum in which the *N*-[^{13}C]methyl resonance centered at 2.78 ppm now appeared as a doublet with the expected ^{13}C-^{1}H coupling constant of 138 Hz. The *N*-[$^2H_3$]methyl derivative (*6*), on the other hand, gave no signal at this position in the ^{1}H-NMR spectrum and yielded an MH$^+$ ion under FAB/MS conditions at *m/z* 368, three mass units higher than the MH$^+$ ion of SMG itself.

Final confirmation of the structures of the glutathione conjugates (*5*)-(*7*) was obtained by FAB/MS/MS, when the spectra of daughter ions recorded upon collision-induced dissociation (CID) of the respective MH$^+$ species were compared with that recorded from the MH$^+$ of unlabelled SMG. In all cases, structurally-informative daughter ions were observed, the proposed origins of which are

consistent with the results of our previous work with such compounds.[2] Loss of the γ-glutamyl moiety (129 u) to give ion *e* represented the major route of fragmentation for all of these glutathione conjugates, and was accompanied by further elimination of the elements of methylisocyanate to yield the prominent ion at *m/z* 179 (*e*-CH_3NCO) which was common to each of the isotopically-enriched analogs of SMG. Appropriate shifts in *m/z* values of the *e* ion for (5)-(7), and a common ion at *m/z* 179 for all three, served to confirm the site of attachment of the isocyanate moiety as being to the cysteinyl thiol. This inference is supported by appropriate shifts in *m/z* ratio for the *a* ion, which arises from elimination of the glycine residue, and by shifts in the *m/z* value for the *g* ions, which also serve to confirm the thiol as the site of modification.

In conclusion, two distinct synthetic routes to methylisocyanate have been adopted to accomplish the convenient and economical preparation of analogs of both methylisocyanate and sulfur conjugates thereof, labelled regiospecifically with ^{13}C, ^{14}C or deuterium. These labelled derivatives are currently being employed to investigate the chemical characteristics and metabolic fate of the glutathione conjugate (SMG) and the corresponding cysteine adduct (SMC) of methylisocyanate.

Table 1. Daughter ion spectra of MH^+ ions from *S*-(*N*-methylcarbamoyl)-glutathione (SMG; *3*) and derivatives thereof (5)-(7).



m/z values and relative abundances of characteristic daughter ions[1]

N°	Substituent (X)	MH+	*a*	*b*	*e*	*f*	*g*	other ions	
(3)	CH_3NCO	365 (20)	290 (2)	75 (18)	236 (99)	130 (6)	133 (100)	179[2] (81)	162[3] (120)
(5)	$^{13}CH_3NCO$	366 (31)	291 (2)	75 (3)	237 (100)	130 (2)	134 (50)	179 (49)	162 (6)
(6)	C^2H_3NCO	368 (30)	293 (3)	75 (3)	239 (100)	130 (2)	136 (50)	179 (44)	162 (5)
(7)	$CH_3N^{13}CO$	366 (22)	291 (3)	75 (2)	237 (100)	130 (2)	134 (54)	179 (47)	162 (8)

1 Ion abundances (in parentheses) are expressed relative to the most intense daughter ion.

2 [e-CH_3NCO]

3 [c-CH_3NCO]

EXPERIMENTAL

High field NMR spectra were obtained on a Varian VXR-300 spectrometer operating at 300 MHz or 75 MHz for ^1H or ^{13}C nuclei, respectively. Samples were dissolved in D_2O and the chemical shifts are expressed in parts per million (δ) downfield from sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) as an internal standard. Signal multiplicities are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Fast atom bombardment (FAB) mass spectra were recorded on a VG-70SEQ hybrid tandem mass spectrometer, of EBQQ geometry (VG Analytical Ltd, Manchester, U.K.), equipped with an Ion Tech fast atom gun and a VG 11/250 data system. Samples (1-5 μg) were dissolved in a thioglycerol matrix containing HCl on a FAB target. Ionization was achieved by bombardment with a primary beam of Xe° (8 keV). Spectra were recorded at an accelerating voltage of 8kV and processed *via* the data system. Isotopic enrichment was calculated by averaging 10 scans across the molecular ion envelope of the analyte. Daughter ion spectra of MH+ species were recorded under FAB/MS/MS conditions, employing low-energy (40 eV) collision-induced dissociation (CID) in the first (rf-only) quadrupole. Argon, employed as a target gas for CID experiments, was maintained at a pressure of 2×10^{-6} Torr. The parent MH+ ions of the analytes were selected by varying the magnetic field strength of the first mass analyzer.

Analytical thin-layer chromatography (TLC) was performed on Analtech silica gel TLC plates (0.25 mm thickness) developed in *n*-propanol:acetic acid:water (100:1:50, by vol.) and the spots were visualized by exposure to iodine vapor.

Radioactivity determinations were performed on a Packard Tricarb liquid scintillation counter operated in the external standard mode.

Glutathione and cysteine were purchased from the Sigma Chemical Co. (St. Louis, MO). Methylisocyanate, silver cyanate, [^{13}C]iodomethane (99 atom % excess), [$^{2}H_3$]iodomethane (99.5 atom % excess), and [1-^{13}C]acetyl chloride (99 atom % excess) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]Iodomethane (30 mCi mmol^{-1}) was obtained from ICN Radiochemicals (Irvine, CA).

S-(*N*-[3-^{13}C]Methylcarbamoyl)glutathione (5) [^{13}C]Iodomethane (440 mg; 3 mmol) was added to a suspension of silver cyanate (500 mg; 3.3 mmol) in acetonitrile and the mixture was stirred at room temperature in the dark for five days. The resulting [3-^{13}C]methylisocyanate was purified by microdistillation, and a portion (70 mg; 1.2 mmol) was dissolved in acetone (2 ml) and added to a solution of glutathione (184 mg; 0.6 mmol) dissolved in 70% acetonitrile in water (10 ml). The mixture was stirred at room temperature (2 hr) and the resultant precipitate was recovered by filtration, and washed with acetonitrile (2 x 5 ml) to afford 175 mg (0.48 mmol) of *S*-(*N*-[3-^{13}C]methylcarbamoyl)glutathione (5) as a white crystalline solid (40%): TLC: R_f 0.52; ^{1}H-NMR (D_2O): 4.63 (1H, dd, J = 9 Hz and 6 Hz; Cys α-CH); 3.96 (2H, s; Gly-CH_2); 3.83 (1H, t, J = 9 Hz; Glu α-CH); 3.44 (1H, dd, J = 15 Hz and 6 Hz) and 3.21 (1H, dd, J = 15 Hz and 9 Hz; Cys β-CH_2); 2.78 (3H, d, J = 138 Hz; *N*-[$^{13}CH_3$]); 2.53 (2H, t, J = 9 Hz; Glu γ-CH_2); and 2.16 (2H, m; Glu β-CH_2). ^{13}C-NMR (D_2O):30.4 (*N*-[$^{13}CH_3$]). FAB/MS: MH$^+$ at *m/z* 366. The isotopic composition of (5) was 97.5 atom % excess [^{13}C] based on the relative abundances of the ions at *m/z* 364-368.

S-(*N*-Trideuteromethylcarbamoyl)glutathione (6) [$^{2}H_3$]Iodomethane (440 mg; 3 mmol) was added to a suspension of silver cyanate (500 mg; 3.3 mmol) in acetonitrile and stirred at room temperature in the dark for five days. The yellow precipitate which formed was removed by filtration, and the filtrate was microdistilled (35-40°C at atmospheric pressure) to yield deuterated

methylisocyanate ([3-$^{2}H_3$]methylisocyanate. The product (72 mg; 1.2 mmol), dissolved in acetone (2 ml), was added to a solution of glutathione (184 mg; 0.6 mmol) in 70% acetonitrile in water (10 ml). The mixture was stirred at room temperature (2 hr) and the resultant precipitate was recovered by filtration, and washed with acetonitrile (2 x 5 ml) to afford 198 mg (0.54 mmol) of *S*-(*N*-trideuteromethylcarbamoyl)glutathione as a white crystalline solid (yield = 45%): TLC: R_f 0.50; ^{1}H-NMR (D_2O): 4.64 (1H, dd J = 9 Hz and 6 Hz; Cys α-CH); 3.97 (2H, s; Gly-CH_2); 3.82 (1H, t, J = 9 Hz; Glu α-CH); 3.43 (1H, dd, J = 18 Hz and 6 Hz) and 3.21 (1H, dd, J = 18 Hz and 9 Hz; Cys β-CH_2); 2.52 (2H, t, J = 9 Hz; Glu γ-CH_2); and 2.15 (2H, m; Glu β-CH_2). FAB/MS: MH+ at *m/z* 368. The isotopic composition of (*6*) was 97.6 atom % excess [$^{2}H_3$] and 2.4 atom % excess [$^{2}H_2$] based on the relative abundances of the ions at *m/z* 364-370.

S-(*N*-[1-^{13}C]Methylcarbamoyl)glutathione (*7*) [1-^{13}C]Acetyl chloride (1 g; 12.6 mmol) was added slowly to a solution of sodium azide (897 mg; 13.8 mmol) in anhydrous toluene, cooled in an ice bath to maintain the temperature below 20°C. The solution was heated under reflux until nitrogen evolution ceased (3 hr) and the product was subsequently microdistilled (35-40°C at atmospheric pressure) to afford [1-^{13}C]methylisocyanate (365 mg; 6.3 mmol). The material thus obtained was reacted with glutathione, as described for the synthesis of (*5*), to afford (*7*) (186 mg; 0.51 mmol) as a white crystalline powder. (Yield = 43%). TLC: R_f 0.52; ^{1}H-NMR (D_2O): 4.64 (1H, dd, J = 7.5 Hz and 5 Hz; Cys α-CH); 3.90 (2H, s; Gly-CH_2); 3.80 (1H, t, J = 7.5 Hz; Glu α-CH); 3.45 (1H, ddd, J = 15 Hz and 5 Hz) and 3.21 (1H, ddd, J = 15 Hz, 7.5 Hz and 5 Hz; Cys β-CH_2); 2.79 (3H, d, J = 5 Hz; S-^{13}CONHCH_3); 2.52 (2H, t, J = 7.5 Hz; Glu γ-CH_2); and 2.16 (2H, m; Glu β-CH_2). ^{13}C-NMR (D_2O):172.0 (*S*-^{13}CO-NHCH_3). FAB/MS: MH+ at *m/z* 366. The isotopic composition of (*7*) was 98.9 atom % excess [^{13}C] based on the relative abundances of the ions at *m/z* 364-368.

S-(*N*-[3-^{14}C]Methylcarbamoyl)glutathione (*8*) A breakseal vial of [^{14}C]iodomethane (1 mCi; 50 mCi/mmol) was paritally immersed in a dry ice/acetone bath for 15 min, acetonitrile (1.5 ml) containing iodomethane (60 µl; 1 mmol) was added to the reservoir, and the seal was broken. The solution was allowed to warm to room temperature and the contents of the vial were transferred to a

round-bottom flask (5 ml) containing slilver cyanate (158 mg; 1.1 mmol). The mixture was stirred in the dark under an argon atmosphere for 5 days, at which time the reaction products were transferred to a 5 ml screw-capped tube and the silver iodide and unreacted silver cyanate were separated by centrifugation at 3000 rpm for 5 min. The clear supernatant containing [3-^{14}C]methylisocyanate was added to a solution of glutathione (30 mg; 1 mmol) in 70% acetonitrile in water (0.6 ml) and stirred for 2 hr. The resultant S-(*N*-[3-^{14}C]methylcarbamoyl)glutathione was recovered as described above for (*5*) and lyophillized to afford (29 mg; 0.8 mmol; 80%; 0.2 mCi mmol^{-1}) of (*8*) as a white powder. The radiochemical purity of (*8*) as determined by TLC analysis was greater than 98%. TLC: R_f 0.51; ^{1}H-NMR (D_2O): 4.65 (1H, dd, J = 9 Hz and 6 Hz; Cys α-CH); 3.90 (2H, s; Gly-CH_2); 3.77 (1H, t, J = 9 Hz; Glu α-CH); 3.44 (1H, dd, J = 15 Hz and 6 Hz) and 3.21 (1H, dd J = 15 Hz and 9 Hz; Cys β-CH_2); 2.79 (3H, s; *N*-$^{14}CH_3$); 2.52 (2H, t, J = 9 Hz; Glu γ-CH_2); and 2.15 (2H, m; Glu β-CH_2). FAB/MS: MH+ at *m/z* 365.

S-(*N*-Trideuteromethylcarbamoyl)cysteine (*9*). Trideuteromethylisocyanate (96 mg; 1.6 mmol), prepared as described in the synthesis of (*6*), was dissolved in acetone (2 ml) and added to a solution of cysteine (100 mg; 0.8 mmol) in 60% acetonitrile in water (10 ml). The solution was stirred for 2 hr, and the resultant precipitate was removed by filtration and washed with dry acetone to afford 116 mg (0.64 mmol) of (*9*) in 40% yield. TLC: R_f 0.59; ^{1}H-NMR (D_2O): 4.04 (1H, dd J = 6.3 Hz and 3.9 Hz; Cys α-CH); 3.54 (1H, dd, J = 14.2 Hz and 3.9 Hz) and 3.32 (1H, dd, J = 14.2 Hz and 6.3 Hz; Cys β-CH_2). FAB/MS: MH+ at *m/z* 182. The isotopic composition of (*9*) was 97.8 atom % excess [$^2H_3$] and 2.2 atom % excess [$^2H_2$] based on the relative abundances of the ions at *m/z* 178-184.

S-(*N*-[3-^{13}C]Methylcarbamoyl)cysteine (*10*). [3-^{13}C]Methylisocyanate (93 mg; 1.6 mmol), prepared as described in the synthesis of (*5*), was reacted with cysteine (100 mg; 0.8 mmol), as described for (*9*), to afford 115 mg (0.64 mmol) of (*10*) (Yield = 40%). TLC: R_f 0.58; ^{1}H-NMR (D_2O): 4.02 (1H, dd, J = 9 Hz and 6 Hz; Cys α-CH); 3.54 (1H, dd, J = 15 Hz and 6 Hz) and 3.32 (1H, dd, J = 15 Hz and 9 Hz; Cys β-CH_2) and 2.78 (3H, d, J = 138 Hz; *N*-$^{13}CH_3$). ^{13}C-NMR (D_2O): 30.4 (*N*-$^{13}CH_3$). FAB/MS: MH+ at *m/z* 180. The precise isotopic composition of (*10*) was

99 atom % excess [^{13}C] based on the relative abundances of the ions at *m/z* 178-182.

S-(*N*-[1-^{13}C]Methylcarbamoyl)cysteine (*11*). [1-^{13}C]Methylisocyanate (93 mg; 1.6 mmol), prepared as described in the synthesis of (*8*), was reacted with cysteine (100 mg; 0.8 mmol), as described for (*9*), to afford 115 mg (0.64 mmol) of (*11*). (Yield = 40%). TLC: R_f 0.58; ^{1}H-NMR (D_2O): 4.06 (1H, dd, J = 7.5 Hz and 5 Hz; Cys α-CH); 3.56 (1H, ddd, J = 15 Hz, 7.5 Hz and 5 Hz) and 3.34 (1H, ddd, J = 15 Hz, 7.5 Hz and 5 Hz; Cys β-CH_2); 2.8 (3H, d, J = 5 Hz; S-^{13}CONHCH$_3$). ^{13}C-NMR (D_2O): 172.0 (-S-^{13}CO-NHCH$_3$). FAB/MS : MH+ at *m/z* 180. The precise isotopic composition of (*11*) was 98.9 atom % excess [^{13}C] based on ions at *m/z* 178-182.

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