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Syntheses of D-Labelled Oxidative Metabolites of Acrylamide and Acrylonitrile for the Quantification of Their Toxicities in Humans

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Syntheses of the labelled oxidative metabolites of acrylamide and acrylonitrile – reference compounds for the evaluation of human exposure to important toxicants – are reported. For that, L-cystine *tert*-butyl ester was acetylated and the product reductively cleaved to L-cysteine *tert*-butyl ester, which reacted with carbamoyl[D_3]oxirane (obtained from [D_3]acrylonitrile and 30% aq. H_2O_2 at pH = 7.0–7.5) and afforded a separable mixture of *tert*-butyl *N*-acetyl-*S*-(2-hydroxy-2-carbamoyl[D_3]ethyl)cysteinate and *tert*-butyl *N*-acetyl-*S*-(1-carbamoyl-2-hydroxy[D_3]ethyl)cysteinate (ca. 9:1). Removal of the *tert*-butyl group in these intermediates with aq. HCl gave the final deuterated internal standards with carbamoyl residues. Protection of the secondary hydroxy group in the major intermediate with *t*BuMe_2SiCl/imidazole in DMF followed by dehydration of the carbamoyl group (trifluoroacetic anhydride/pyridine in CH_2Cl_2) and stepwise removal of the *tert*-butyl and *t*BuMe_2Si protecting groups (TFA, Et_3SiH, CH_2Cl_2; aq. HF in MeCN) yielded *N*-acetyl-*S*-(2-cyano-2-hydroxy[D_3]ethyl)cysteine. Monoprotection of [D_4]ethylene glycol with *t*BuMe_2SiCl and NaH in THF, oxidation to *t*BuMe_2SiOCD_2CDO, conversion to *t*BuMe_2SiOCD_2CD(OH)CN and *t*BuMe_2SiOCD_2CD(OTs)CN followed by nucleophilic substitution of the tosyloxy group with *N*-acetyl-L-cysteine (MeOD, Et_3N) and deprotection with 4 M HCl in dioxane resulted in *N*-acetyl-*S*-(1-cyano-2-hydroxy[D_3]ethyl)cysteine. All transformations (except the last but one) gave the respective products in good yields.

Introduction

Acrylonitrile (AN) and acrylamide (AA) are industrially produced worldwide in amounts of 6 million and 100 thousand tons per year, and approximately 1% of these amounts is lost polluting the environment. Acrylamide is also formed from asparagine and carbohydrates[1a–1g] or from alanine-containing peptides (without carbonyl compounds)[1h,1i] upon production and heating of food, such as French fries, potato chips, popcorn, coffee, bread, cereals, etc. AA and AN are components of cigarette smoke.[2] AA is neurotoxic for humans,[3] carcinogenic[4,5] and has genotoxic, neurotoxic and reproductive hazardous effects.[5] AN was found to be carcinogenic in rats[6] and mutagenic in E. coli.[7] It is also toxic in cardiovascular (blood), development, gastrointestinal (liver), immuno, neuro, reproductive and respiratory systems, and acts as a skin or sense organ toxicant. Due to their high reactivities, AA and AN show complex metabolisms. As they are good Michael acceptors, they readily react with glutathione (GSH), furnishing the corresponding mercapturic acids [L-AcNHCH(COOH)-$CH_2SCH_2CH_2E$, E = $CONH_2$ or CN]. Another type of metabolism – epoxidation to glycidamide (GA) and glycidonitrile (GN) – is catalyzed by the Cytochrome P450 2E1 enzyme.[8] These poisonous intermediates are believed to be responsible for the carcinogenic activity of the parent compounds, and therefore the quantitative determination of these oxiranes is very important. In vivo, oxiranes can alkylate such nucleophiles as the N-terminus of haemoglobin, DNA, cysteine-containing enzymes or GSH.[9] Conjugation of GA and GN with nucleophilic sites is accomplished by the opening of the oxirane ring activated with carbamoyl or cyano electron-acceptor groups. A priori, it is not easy to predict the regioselectivity of this reaction. Electronic and steric effects in the starting oxiranes oppose each other. In vitro, a similar reaction of MeSH with oxiranes obtained from CH_2=CHR (R = COMe, COOMe, $CONH_2$, COONa and Me) always gives mixtures of two products. The content of the regioisomer derived from the α-attack varies in this sequence from nearly 100 to 0%.[10]

GSH conjugates of AA and AN are excreted in urine as the corresponding mercapturic acids (*S*-alkylated *N*-acetyl-L-cysteines), after enzymatic cleavage of the glutamine and glycine residues from glutathione and acetylation of the amino group of the cysteine moiety. In humans, only one oxidative metabolite of AA has been found, which arises

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from a β-attack of the SH-group on the unsubstituted carbon atom of the oxirane ring in GA (compound **4**-H).[11] The other regioisomer was not detected in this study,[11] as well as in the simultaneously published report, in which the labelled compound **4**-D was used as an internal standard for the determination of GA in human urine.[12] Yet, no experimental details of the preparation of the labelled oxidative metabolites of AA have been reported. In another study, in which [$^{13}C_3$]-AA (alone or along with [$^{13}C_3$]-AN) was administered to mice and rats, and the composition of a complex mixture of metabolites was analyzed by ^{13}C NMR spectroscopy, the same regioisomer ([$^{13}C_3$]-**4**-H) was detected, and, additionally, some ^{13}C NMR signals (C*) were attributed to the "minor" metabolite ([$^{13}C_3$]-**5**-H).[13] These results indicate, that for the quantification of the oxidative metabolites of AA, it is necessary to develop a practical synthesis of both isotopically labelled mercapturic acids **4** and **5**.[14] Isotopic labelling allows one to use these deuterated analyte analogues as "ideal" internal standards when employing the method of isotopic dilution, in which the quantitative results do not depend on the conditions of sample preparation.

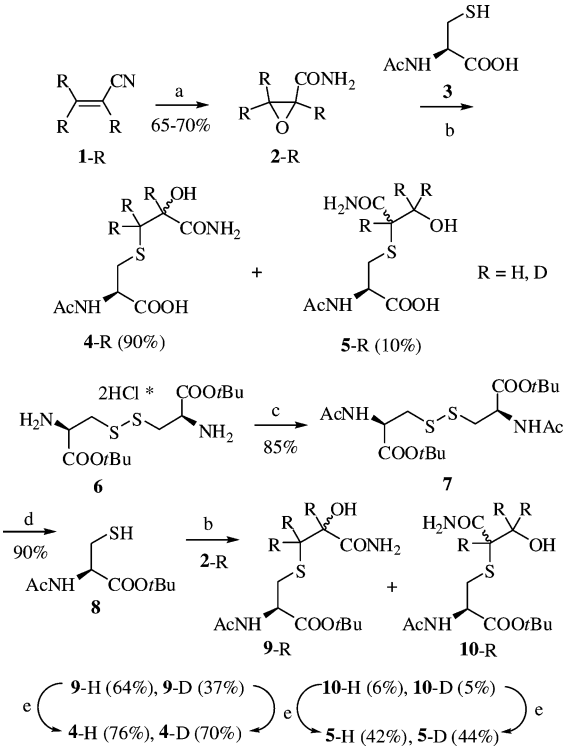
Similar metabolism studies of acrylonitrile and [$^{13}C_3$]-AN were conducted only with mice or rats (not with humans).[8,15] In 1988 Linhart et al. reported that the conjugation of GN with GSH in vivo (rats) does not proceed regioselectively, and both α- as well as β-carbon atoms of GN are attacked by the nucleophilic thiol group.[15] However, the primary product of the β-attack was not detected, but its stable secondary metabolite [L-AcNHCH(COOH)-$CH_2SCH_2CH_2OH$] was identified. Later this result was confirmed by another group. It was found that the metabolism of [$^{13}C_3$]-AN via the epoxide in mice and rats leads to the stable product of an α-attack ([$^{13}C_3$]-**13**-H) which is formed as a mixture of two epimers, as well as in several other substances which presumably arise from the unstable primary product of a β-attack.[8] It is not clear, why the primary product of the β-attack is unstable in vivo. In vitro, the reaction of methyl *N*-acetylcysteinate with GN (under basic conditions) gives predominantly the product of the β-attack (methyl ester of **12**-H).[15] Quantitative data have not been reported, and earlier the same group stated that this regioisomer had been formed exclusively.[16] To clarify the product distribution in the oxidative metabolism of AN, it was necessary to synthesize both regioisomeric ring-opening products from glycidonitrile and *N*-acetylcysteine along routes, which do not leave any doubt about their structures and allow one to prepare them in isotopically labelled forms as internal standards for determination and quantification of the corresponding urinary metabolite(s) in rodents and humans.[17]

Results and Discussion

In modern analytical procedures, the metabolites are separated by HPLC and detected by ESI tandem mass spectrometry in positive or negative ionization mode. If labelled internal standards are used as additives to urine, and the metabolites are not chemically transformed during the sample preparation and analysis, the mass-spectrometric fragmentation pattern diagnostic of the nature of the observed charged species determines the required positions of the isotopic labels in the additives. LC-MS/MS spectra of the oxidative metabolites of AA and AN are simpler in the negative ionization mode, and – at least in the case of the main oxidative metabolite of acrylamide[12] – the main peak corresponds to the negatively charged fragment $SCH_2CH(OH)CONH_2$. Therefore, it was necessary to synthesize the compounds with stable isotopes in their acrylamide and acrylonitrile fragments, respectively.

Epoxidation of acrylonitrile and the commercially available [D_3]acrylonitrile with 30% aq. H_2O_2 at pH = 7.0–7.5 according to a published method furnishes the glycidamide and its deuterated analogue **2**-D in 65–70% yield.[18] Reaction of **2**-D with *N*-acetylcysteine (**3**) under basic conditions gave a mixture of the two regioisomeric mercapturic acids **4** and **5** in a ratio of ca. 9:1, which could not be separated by simple chromatography on silica gel (Scheme 1), but by preparative reversed-phase (RP) HPLC. Each of the compounds **4** and **5** was thus isolated as a mixture of two epimers from the complex crude product, which also contained 2,3-dihydroxypropanamide, glyceric acid, traces of the starting material, hydrogen peroxide, which oxidized *N*-acetylcysteine to *N*-acetylcystine, and other unidentified substances. However, starting with *tert*-butyl *N*-acetylcysteinate (**8**), which can easily be prepared from commercial *tert*-butyl cystinate (**6**) by *N*-acetylation with acetic anhydride and subsequent reductive cleavage of the disulfide bond with sodium borohydride (other reducing agents such as Zn in AcOH or Ph_3P in refluxing aq. EtOH gave inferior results), led to a crude product from which the *tert*-butyl esters of the regioisomeric mercapturic acids **9** and **10** could be isolated by ordinary column chromatography on silica gel.

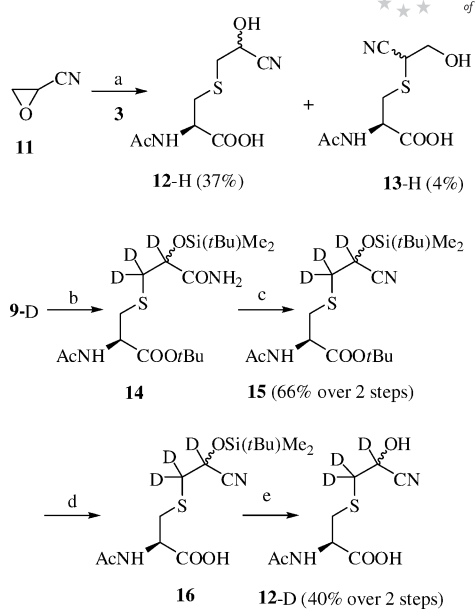
The *tert*-butyl ester groups in the hydroxyamides **9** and **10** could easily be cleaved by treatment with 10% hydrochloric acid at 0 °C to furnish the free acids **4** and **5**, respectively. The hydroxyamide residues in compounds **4** and **5** are stable enough under the reaction conditions and during workup, but for prolonged storage, the final mercapturic acids **4** and **5** should be purified by ordinary chromatography or RP-HPLC in order to remove any traces of a strong acid. When performing the addition of **8** to the epoxide **2**-R at pH ≈ 9 in a mixture of acetonitrile and aq. $Na_2CO_3/NaHCO_3$, the ratio of the regioisomeric adducts **4/5** was found to be ca. 9:1. In the case of MeSH at pD = 9.8, the products of the α-/β-attack on glycidamide **2**-H were formed in a ratio of ca. 22:78,[10] i.e. the α-adduct was obtained to a smaller extent than the β-adduct in this study. At pH ≥ 8, the thiolate ion of cysteine (pK_a = 8.2) is in fact the reactive nucleophile. At physiological pH values (7.8–8.3) the selectivity of glutathione towards GA may even be higher. This may explain why the minor oxidative metabolite of GA has not yet been detected in vivo.[11,12]



Scheme 1. Preparation of the oxidative metabolites of acrylamide (**4**-H, **5**-H) and internal standards (**4**-D, **5**-D) from acrylonitrile and [D_3]acrylonitrile, respectively: (a) 30% aq. H_2O_2, aq. NaOH, pH = 7.3–7.8; (b) aq. Na_2CO_3, MeCN, pH = 8–9; (c) Ac_2O, pyridine, 0 °C; (d) $NaBH_4$, EtOH; (e) aq. HCl, THF, 0 °C.

Labelled oxidative metabolites of acrylonitrile would be accessible analogously by the nucleophilic addition of *N*-acetylcysteine (**3**) to glycidonitrile, provided this oxirane would be available in its deuterated form. The undeuterated glycidonitrile (GN) **11**-H reacted with *N*-acetylcysteine (**3**) to produce the regioisomeric mercapturic acids **12**-H/**13**-H in a ratio of ca. 9:1 (Scheme 2).

Unfortunately, the only practical synthesis of the highly reactive cyanooxirane **11** requires a very large excess of acrylonitrile (**1**-H): from 200 mL only ca. 5 g of **11** is obtained in its reaction with an aqueous solution of sodium hypochlorite,[19] and the excess of **1**-H is distilled off in vacuo through a long Vigreux column. Thus, from 1 g of [D_3]-AN only 50 mg of [D_3]-GN may be prepared at best, and it is rather difficult to completely remove unreacted **1**-D from the few drops of the product [D_3]-GN. An attempt to use a larger amount of sodium hypochlorite on [D_3]-AN did not help much, and after careful evaporation of the starting material **1**-D and addition of the estimated equimolar amount of *N*-acetylcysteine (**3**) under basic conditions, only a trace of the major of the two regioisomeric products in its deuterated form **12**-D could be detected by HPLC, along with the unreacted *N*-acetylcysteine. Because the secondary kinetic isotopic effect of the deuterium atoms may somewhat reduce the epoxidation rate, the epoxidation of **1**-D was also attempted with trifluoroperacetic acid, which was prepared in situ from trifluoroacetic anhydride



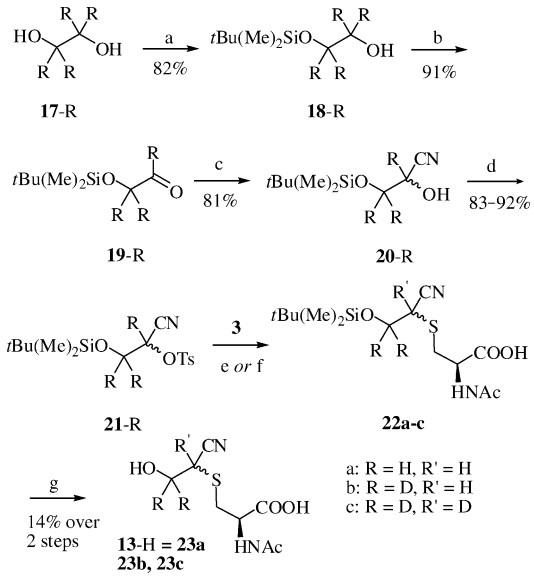
Scheme 2. Preparation of the oxidative metabolites of acrylonitrile **12**-H, **13**-H and the internal standard **12**-D from cyanooxirane and the deuterated hydroxyamide **9**-D, respectively: (a) aq. Na_2CO_3, MeCN, room temp.; (b) *t*$BuMe_2SiCl$, DMF, imidazole, 5 °C, 4 d; (c) $(CF_3CO)_2O$, pyridine (2 equiv.), CH_2Cl_2, room temp., 16 h; (d) Et_3SiH, TFA, CH_2Cl_2, 5 °C, 2 d; room temp. 1 d; (e) 51% aq. HF, MeCN, 2 h, room temp.

(TFAA) and 90% aq. H_2O_2 in a phosphate buffer. Even when carrying out this reaction in refluxing CH_2Cl_2, no product could be detected, and only the starting material **1**-D was recovered. An attempted dehydration of the crude glycidamide **2**-D with TFAA/pyridine was also unsuccessful.

Therefore, an alternative approach to the deuterated hydroxynitriles **12**-D and **13**-D had to be developed. The synthesis of the labelled major oxidative metabolite of acrylonitrile turned out to be straightforward (Scheme 2). Towards that, the hydroxy group in the amide **9**-D was first protected as a *tert*-butyldimethylsilyl ether, and then the amide was dehydrated to a nitrile by treatment with TFAA/pyridine in CH_2Cl_2. After some screening of conditions, the *tert*-butyl ester and the silyl ether were cleaved in two steps. The *tert*-butyl group was removed by treatment with TFA and Et_3SiH as a cation scavenger in anhydrous CH_2Cl_2. The dimethylsilyl group was cleaved off by the addition of aq. HF to an acetonitrile solution of the concentrated reaction mixture from the previous step. Finally, the hydroxynitrile **12**-D was purified by preparative HPLC.

A reliable synthesis of the minor oxidative metabolite of AN started from ethylene glycol **17**-H (Scheme 3), which is also commercially available in the tetradeuterated form **17**-D. The diol was protected on one hydroxy group as a *tert*-butyldimethylsilyl ether, and the alcohol **18**-R[20] was oxidized to the aldehyde **19**-R,[21] which was converted into the cyanohydrine **20**-R. The latter was tosylated, and then the tosyloxy group in **21**-R was replaced by the thiol group of *N*-acetylcysteine (**3**) in the presence of an excess of Et_3N in methanol. The yields in all steps, except the last one, were

good. The low yield (25–30%) of the desired product **22** may be due to the bis(nucleophilic) character of the reagent **3** and the bis(electrophilic) nature of the substrate **21**-R, which may lead to substitution of both the tosyloxy and the cyano leaving groups with formation of a six-membered lactone ring. The use of the aprotic solvent DMF did not improve the yield. However, the simplicity of the procedure and the isolation of the product make this protocol more convenient than the two-step variant employing *tert*-butyl *N*-acetylcysteinate **8** for the substitution and subsequent removal of the *tert*-butyl protecting group.[22] After workup, the crude product mixture was distributed between diethyl ether and aqueous sodium hydrogen carbonate solution. The latter, which contained the carboxylic acid **22** and the excess *N*-acetylcysteine (**3**), was carefully acidified with citric acid. Subsequent extraction with diethyl ether left *N*-acetylcysteine in the aqueous phase; yet some of the product **22** may be lost during acidification, because the silyl ether may be cleaved to a certain extent even at pH = 2–3. The deuterium label adjacent to the cyano group (in **22**) is prone to deuterium/hydrogen exchange in the presence of a base in a protic solvent, as the neighboring sulfur atom also stabilizes an anionic center adjacent to it. Therefore, under the employed conditions, the product **22b** with only two deuterium labels was obtained. When MeOD was used as a solvent instead of MeOH, and Na_2CO_3 in D_2O for the workup, as well as D_2SO_4 in D_2O for the acidification, the deuterated product **22c** was indeed obtained.



Scheme 3. Total synthesis of the oxidative metabolites of acrylonitrile **1**-H(D) in the unlabelled (**13**-H) and labelled form (**23b**–**c**): (a) NaH, THF; *t*BuMe$_2$SiCl; (b) (COCl)$_2$, DMSO, –78 °C; Et$_3$N, room temp.; (c) $Na_2S_2O_5$, KCN, aq. MeOH; (d) *p*-MeC$_6$H$_4$SO$_2$Cl (TsCl), pyridine; (e) MeOH/(f) [D]MeOH, Et$_3$N, 40 °C 36 h; (g) 4 M HCl in dioxane.

Finally, the *tert*-butyldimethylsilyl protecting group in **22a**–**c** was removed by treatment with 4 M HCl in dioxane, and the mercapturic acids **23a**–**c** were purified either by conventional silica gel chromatography or RP-HPLC. With both labelled oxidative metabolites **12**-D and **23c** of acrylonitrile at hand, it will be easy not only to prove the results of Sumner et al., who claimed that, after administration of [1,2,3-$^{13}C_3$]-AN to rats and mice, only one oxidative metabolite, namely [1,2,3-$^{13}C_3$]-**23**, had been excreted with urine,[8,13] but also to quantitatively determine its or their content in urine. The structure of the latter metabolite was assigned on the basis of the proton multiplicities of ^{13}C NMR signals, which were determined by heteronuclear 2D *J*-resolved spectroscopy (HET2DJ). The carbon–carbon connectivities were established with the INADEQUATE technique. Resonances were assigned on the basis of chemical shifts, proton multiplicities, values of the ^{13}C-^{13}C coupling constants and calculated values of the chemical shifts.[8b] For a rigorous proof, a model (unlabelled) compound was synthesized according to the method of Linhart et al.[15,17] For comparison, the diagnostically important ^{13}C NMR chemical shifts (in [D$_6$]DMSO) for the major diastereomer **12** are δ = 36.3 and 61.2/61.3 ppm for the SCH_2 and CH(OH)CN groups, respectively (in the two epimers). For the minor diastereomer **23** the CH(CN)S and CH_2OH groups have the chemical shifts of δ = 35.4/35.5 and 61.8/61.9 ppm, respectively. Sumner et al. were aware of this similarity,[8b] but stated that the carbon–carbon couplings and proton multiplicities are consistent with the *S*-(1-cyano-2-hydroxyethyl)cysteine derivative [1,2,3-$^{13}C_3$]-**23**. Therefore, it is crucial to compare the ^{1}H NMR chemical shifts of Linhart's compound (presumably **23a**),[15,17] which were measured in [D$_6$]acetone, with the values obtained in this study. Esterification of both mercapturic acids **12**-H and **23a** with diazomethane and subsequent measurement of their ^{1}H NMR spectra unequivocally confirmed the results of Linhart et al. in that only the minor regioisomer **23a** gave a methyl ester with the same spectrum as the reported one.[23]

The major regioisomer resulting from a β-attack of *N*-acetylcysteine on cyanooxirane prepared in this study (compound **12**-H/D), which previously had not been detected in vivo and thought to be unstable in rodents,[8,13b,15] may be used in the unlabelled and labelled form to clarify, if any stable secondary metabolites are going to be formed from it in vivo. Towards that, new urine metabolites have to be found by means of LC-MS, after administration of the hydroxynitrile **12**-H/D to rodents.

Conclusion and Outlook

Practical syntheses of all four oxidative metabolites of acrylamide and acrylonitrile in their unlabelled and deuterium-labelled forms, which can be reproduced in all toxicological and analytical laboratories, make it possible to further study the metabolism of acrylamide and acrylonitrile and quantify human exposure to these toxic electrophiles. An approach to compounds with the rare structural fragment $R^1OCH_2CH(SR^2)CN$ [R^1 = protecting group or H, R^2 = (functionally substituted) alkyl or aryl group] has been developed.

Experimental Section

General: NMR spectra were recorded with Varian MERCURY 300 and Bruker AM 250 spectrometers at 300 (^{1}H) and 75.5 MHz (^{13}C and APT), as well as at 250 (^{1}H) and 62.9 MHz (^{13}C and DEPT), respectively. All spectra are referenced to tetramethylsilane as an internal standard (δ = 0 ppm) by using the signals of the residual protons of $CHCl_3$ (δ =7.26 ppm) in $CDCl_3$, CHD_2OD (δ =3.31 ppm) in CD_3OD, HOD (δ =4.79 ppm) in D_2O or [D_5]DMSO (δ =2.50 ppm) in [D_6]DMSO. Multiplicities of signals are described as follows: br. = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, m_c = centrosymmetrical multiplet. Coupling constants (J) are given in Hz. Multiplicities in the ^{13}C NMR spectra were determined by DEPT or APT (Attached Proton Test) measurements. Isotopic purity of all deuterated compounds was found to be >95% by ^{13}C NMR spectroscopy and mass spectrometry. Low-resolution mass spectra (electrospray ionization, ESI) were obtained with an LCQ spectrometer. High-resolution mass spectra (ESI-HRMS) were obtained with an APEX IV spectrometer. HPLC: 20- and 100-μL injection loops for the analytical and preparative columns, respectively; analytical column: Eurospher-100 C18, 5 μm, 250 × 4 mm, 1 mL/min; preparative column: Eurosphere-100 C18, 5 μm, 250 × 8 mm, 4 mL/min; 25 °C, solvent A: water + 0.1% v/v trifluoroacetic acid (TFA); solvent B: MeCN + 0.1% v/v TFA; detection at 220 nm. Elemental analyses were carried out in the Mikroanalytisches Laboratorium des Instituts für Organische und Biomolekulare Chemie der Universität Göttingen. Analytical TLC was performed on Merck ready-to-use plates with silica gel 60 (F_{254}), detection under UV light at 254 nm and development with MOPS reagent (10% molybdophosphoric acid in ethanol). Column chromatography: Merck silica gel, grade 60, 0.04–0.063 mm. Organic solutions were dried with anhydrous Na_2SO_4. All reactions were carried out with magnetic stirring.

Reaction of *N*-Acetylcysteine (3) with Glycidamide (2-H). *N*-Acetyl-*S*-[(*R*/*S*)-2-carbamoyl-2-hydroxy]ethylcysteine (4-H) and *N*-Acetyl-*S*-[(*R*/*S*)-1-carbamoyl-2-hydroxyethyl]cysteine (5-H): A suspension of *N*-acetylcysteine (**3**, 16.3 mg, 0.10 mmol) in water (0.3 mL) was flushed with argon, cooled to 4 °C, then 20% aq. Na_2CO_3 (50 μL) and subsequently the glycidamide **2**-H (17 mg, 0.20 mmol) were added. The reaction mixture was warmed up to room temperature with rapid stirring and, after 4 h, acidified with 1 M aq. HCl to pH = 2. The compounds **4**-H and **5**-H were detected by HPLC (column 4 × 250 mm; A/B, 99:1; isocratic mode) in the complex reaction mixture with the help of reference samples, which were prepared and characterized as described below. Compound **5**-H: t_R = 4.5 and 4.7 min (2:3, 2 epimers of the minor regioisomer); compound **4**-H: t_R = 6.0 min (for both epimers of the major regioisomer).

[D_3]Glycidamide (2-D): Compound **2**-D was obtained from freshly distilled [D_3]acrylonitrile (1.0 g, 18 mmol) in H_2O (7.0 mL) and 30% aq. H_2O_2 (1.9 g, 17 mmol) by adding 1 M aq. NaOH (ca. 0.5 mL) at such a rate that the pH value was maintained at 7.3–7.8 (pH meter!).[18] Unreacted H_2O_2 was destroyed by adding 10% Pd/C (ca. 50 mg) to the reaction mixture, when the consumption of NaOH ceased, and the pH value did not return to 7.3 anymore. After standing at +4 °C overnight, the reaction mixture was centrifuged, and the supernatant solution was used for the reaction with *N*-acetylcysteine (**3**) (see: preparation and HPLC isolation of the compounds **4**-D and **5**-D) and for the synthesis of preparatively useful amounts of the esters **9** and **10**.

Di-*tert*-butyl *N*-Acetylcystinate (7): Acetic anhydride (2.7 mL, 29 mmol) was added in one portion to a suspension of di-*tert*-butyl cystinate dihydrochloride (**6**) (2.48 g, 5.83 mmol) in anhydrous pyridine (25 mL), cooled to 0 °C. The reaction flask was shaken for several minutes, and the solid dissolved while an exothermic reaction took place. The reaction mixture was left at +4 °C for 2 d, then ice (100 g) was added, followed by concd. aq. HCl (25 mL), which was introduced in small portions with stirring, so that the temperature did not exceed 20 °C and the pH value remained above 4–5. The reaction mixture was extracted with diethyl ether (4 × 100 mL); the combined organic solutions were washed with cold 0.5 M aq. H_2SO_4, until the pH value of the aqueous layer reached 2–3, and subsequently with satd. aq. $NaHCO_3$. After drying and evaporation of the solvent, the residue was dissolved in hot $CHCl_3$ (20 mL), and hexane was added, until the solution became turbid. The mixture was left at +4 °C for 16 h, the colorless precipitate was collected by filtration to give 2.16 g (85%) of **7** with m.p. 80–81 °C (diethyl ether). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.43 (s, 9 H), 2.00 (s, 3 H), 3.14 (d, J = 5.1 Hz, 2 H), 4.71 (m_c, 1 H), 6.69 (br.d, J = 7.3 Hz, 1 H, NH) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 23.0 (Me), 27.9 (Me × 3), 41.4 (CH_2), 52.5 (CH), 82.9 (C-O), 169.4 and 170.0 (CO) ppm. MS (ES): *m*/*z* (positive mode, %) = 895 (100) [2 M + Na]$^+$, 459 (69) [M + Na]$^+$; *m*/*z* (negative mode, %) = 481 (100) [M + $HCOO^-$]. $C_{18}H_{32}N_2O_6S_2$ (436.6): calcd. C 49.52, H 7.39, N 6.41; found C 49.63, H 7.39, N 6.41.

***tert*-Butyl *N*-Acetylcysteinate (8):** $NaBH_4$ (1.78 g, 49.5 mmol) was added in small portions to a solution of compound **7** (2.16 g, 4.95 mmol) in anhydrous EtOH (100 mL) with cooling at 20 °C. The cooling bath was removed, and the mixture was left to warm up to ca. 30 °C. An exothermic reaction occurred, and the EtOH eventually started to boil. After it ceased, an amorphous solid separated; $CHCl_3$ (200 mL) and 5% aq. HCl (50 mL) were carefully added to the reaction mixture with cooling by ice/water. The organic layer was separated, washed with water (100 mL), brine (50 mL), dried and concentrated to yield **8** (1.95 g, 90%) as an oil, which slowly crystallized at +4 °C to a colorless low-melting solid. It was readily oxidized by air oxygen (to compound **7**) and was therefore kept under an inert gas. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.28 (t, J = 8.9 Hz, 1 H, SH), 1.42 (s, 9 H), 1.99 (s, 3 H), 2.92 (m_c, 2 H), 4.71 (dt, J = 3.5 and 4 Hz, 1 H), 6.69 (br. d, J = 7 Hz, 1 H, NH) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 23.0 (Me), 26.9 (CH_2), 27.8 (Me, ×3), 53.7 (CH), 82.7 (C-O), 169.1 and 169.8 (CO) ppm. MS (ES): *m*/*z* (positive mode, %) = 461 (19) [2 M + Na]$^+$, 242 (100) [M + Na]$^+$; *m*/*z* (negative mode, %) = 218 (41) [M – H]$^-$.

***tert*-Butyl *N*-Acetyl-*S*-[(*R*/*S*)-2-carbamoyl-2-hydroxyethyl]cysteinate (9-H) and *tert*-Butyl *N*-Acetyl-*S*-[(*R*/*S*)-1-carbamoyl-2-hydroxyethyl]cysteinate (10-H):** Glycidamide **2**-H (200 mg, 2.3 mmol, as a waxy solid),[18] then water (10 mL) and 20% aq. Na_2CO_3 (0.5 mL) were added to a solution of compound **8** (450 mg, 2.05 mmol) in MeCN (20 mL), kept under argon. After 1 h, TLC (CH_2Cl_2/MeOH, 10:1) disclosed that the starting thiol had been fully consumed, and two new compounds with $R_f \approx 0.30$ (major) and 0.27 (minor) appeared. The reaction mixture was diluted with EtOAc (100 mL), the organic layer was separated, washed with brine and concentrated in vacuo to give an oily residue (1.3 g). The latter was subjected to chromatography on SiO_2 (120 g), eluting with CH_2Cl_2/MeOH (12:1) to give two regioisomeric products; 400 mg (64%) of **9**-H as an oil, 40 mg (6.4%) of **10**-H as an oil. **9-H:** ^{1}H NMR (250 MHz, $CDCl_3$, 2 diastereomers): δ = 1.40 (s, 9 H), 1.98 (s, 3 H), 2.72–3.10 (m, 4 H, CH_2S), 4.20 (m_c, 1 H, C*H*OH), 4.62 (m_c, 1 H, C*H*NH), ca. 5.4 (br. s, 1 H, OH), 6.70 (br. s, 1 H, $CONH_2$), 7.10 (br. s, 1 H, $CONH_2$), 7.21 (d, J = 7 Hz, 1 H, N*H*Ac) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$, 2 diastereomers): δ = 22.8 (Ac), 27.7 (Me, ×3), 35.2 (CH_2S), 37.7 (CH_2S), 52.9/53.0 (C*H*NH), 70.8/71.1 (C*H*OH), 82.77/82.83 (C-O), 169.8, 170.9 and 175.97/176.00 (CO)

ppm. MS (ES): *m*/*z* (positive mode, %) = 635 (32) $[2\ M + Na]^+$, 329 (100) $[M + Na]^+$. **10-H:** ^1H NMR (250 MHz, $CDCl_3$, 2 diastereomers): δ = 1.42 (s, 9 H), 2.05/2.07 (s, Σ 3 H), 2.82–3.15 (m, 2 H, CH_2S), 3.50 (m, 1 H, CHS), 3.88 (m, 2 H, CH_2O), 4.69 (m, 1 H, C*H*NH), ca. 4.8 (br. s, 1 H, OH), 6.49/6.62 (br. s, Σ 1 H, $CONH_2$), 6.96/7.05 (d, J = 7 Hz, Σ 1 H, N*H*Ac), 7.11/7.27 (br. s, Σ 1 H, $CONH_2$) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$, 2 diastereomers): δ = 22.97/23.05 (Ac), 27.9 (Me, ×3), 33.9/34.4 (CH_2S), 49.2/50.2 (CHS), 52.4/52.9 (C*H*NH), 62.6/62.8 (C*H*$_2$OH), 83.2/83.3 (C-O), 169.65/169.71, 170.9/171.1 and 174.05/174.11 (CO) ppm.

***tert*-Butyl *N*-Acetyl-*S*-{(*R*/*S*)-2-carbamoyl-2-hydroxy[1,1,2-D$_3$]ethyl}-cysteinate (9-D) and *tert*-Butyl *N*-Acetyl-*S*-{(*R*/*S*)-1-carbamoyl-2-hydroxy[1,2,2-D$_3$]ethyl}cysteinate (10-D):** The *tert*-butyl ester **8** (1.8 g, 8.2 mmol) was added under argon to an aqueous solution (ca. 5 mL) of [D_3]glycidamide (**2**-D, ca. 700 mg, 7.8 mmol), followed by MeCN (20 mL) and 20% aq. Na_2CO_3 (0.5 mL). The mixture was stirred rapidly under argon for 4 h, and the title compounds were isolated as described above for the unlabelled analogues; 0.90 g (37%) of **9**-D as an oil, 0.13 g (5.4%) of **10**-D as an oil. **9-D:** ^1H NMR (250 MHz, $CDCl_3$, 2 diastereomers): δ = 1.46 (s, 9 H), 2.03 (s, 3 H), 2.84–3.08 (m_c, 2 H, CH_2S), 4.69 (m_c, 1 H, C*H*NH), 4.98/5.08 (s, Σ 1 H, OH), 6.33 (br. s, 1 H, $CONH_2$), 6.90 ("t", J = 7 Hz, 1 H, N*H*Ac), 7.03 (br. s, 1 H, $CONH_2$) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$, 2 diastereomers): δ = 23.1 (Ac), 27.9 (Me ×3), 35.5 (CH_2S), 53.0/53.4 (C*H*NH), 83.1/83.2 (C-O), 169.8, 170.9 and 175.5 (CO) ppm. **10-D:** ^1H NMR (250 MHz, $CDCl_3$, 2 diastereomers): δ = 1.43 (s, 9 H), 2.02/2.04 (s, Σ 3 H), 2.88–3.13 (m, 2 H, CH_2S), 4.3/4.4 (br. s, Σ 1 H, OH), 4.68 (m_c, 1 H, C*H*NH), 6.53/6.67 (br. s, Σ 1 H, $CONH_2$), 7.09 (br. m, 1 H, N*H*Ac), 7.17/7.27 (br. s, Σ 1 H, $CONH_2$) ppm. ^{13}C NMR (62.9 MHz, $CDCl_3$, 2 diastereomers): δ = 22.9/23.0 (Ac), 27.9 (Me, ×3), 33.6/34.1 (CH_2S), 52.5/52.9 (C*H*NH), 83.1/83.2 (C-O), 169.66/169.73, 170.9/171.0 and 174.0 (CO) ppm. MS (ES): *m*/*z* (positive mode, %) = 641 (100) $[2\ M + Na]^+$, 332 (82) $[M + Na]^+$; *m*/*z* (negative mode, %) = 354 (100) $[M + HCOO^-]$.

***N*-Acetyl-*S*-[(*R*/*S*)-2-carbamoyl-2-hydroxyethyl]cysteine (4-H):** Concd. aq. HCl (1.5 mL) was added dropwise with stirring to an ice-cold solution of the *tert*-butyl ester **9**-H (179 mg, 0.585 mmol) in water (3 mL). The reaction mixture was kept at +4 °C for 2 h, the aq. HCl was evaporated in vacuo (1–2 mbar), and the residue was purified by chromatography on SiO_2 (50 g) eluting with a PrOH/water/AcOH (9:1:1) mixture; $R_f \approx 0.5$, developed with aq. $KMnO_4$. Yield: 112 mg (76%) of a colorless glassy solid; t_R = 6.0 min; A/B, 99:1; isocratic mode. ^1H NMR (300 MHz, [D_6]-DMSO, 2 diastereomers): δ = 1.85 (s, 3 H) ppm; ABX system (SC*H*$_2$C*H*OH): δ_A = 2.67/2.69, δ_B = 2.81/2.83, δ_X = 3.98 (J_{AB} = 13.6, J_{AX} = 7.3, J_{BX} = 4) ppm; ABX system (SC*H*$_2$C*H*NH): δ_A = 2.77/2.78, δ_B = 2.95/2.96, δ_X = 4.36 (J_{AB} = 13.6, J_{AX} = 8.3, J_{BX} = 5.1) ppm; δ = ca. 5.8 (br. s, OH), 7.2 (br. s, 2 H, $CONH_2$), 8.20 (d, J = 6 Hz, 1 H, NH) ppm. ^{13}C NMR (75.5 MHz, [D_6]DMSO, 2 diastereomers): δ = 22.4 (Ac), 33.9/34.0 (*C*H$_2$CHNH), 36.75/36.83 (*C*H$_2$CHOH), 52.2/52.3 (C*H*NH), 71.3/71.4 (C*H*OH), 169.4, 172.2, 174.7/174.8 (CO) ppm. MS (ES): *m*/*z* (positive mode, %) = 790 (58) $[3\ M + NH_3 + Na]^+$, 523 (67) $[2\ M + Na]^+$, 273 (56) $[M + Na]^+$, 251 (100) $[M + H]^+$; *m*/*z* (negative mode, %) = 499 (100) $[2\ M - H]^-$, 249 (67) $[M - H]^-$. HRMS ($C_8H_{14}N_2O_5S$): *m*/*z* = 251.06981 [M + H] {251.06962 (calcd.)}, 273.05177 [M + Na] {273.05156 (calcd.)}.

***N*-Acetyl-*S*-{(*R*/*S*)-2-carbamoyl-2-hydroxy[1,2,2-D$_3$]ethyl}cysteine (4-D):** Compound **4**-D was obtained from the *tert*-butyl ester **9**-D (276 mg, 0.89 mmol) in water (3 mL) as described above for the corresponding unlabelled compound **4**-H (1.5 mL of concd. aq. HCl, 0 °C, 2 h). Yield: 158 mg (70%) as a colorless foam. ^1H NMR (300 MHz, [D_6]DMSO, 2 diastereomers): δ = 1.84 (s, 3 H) ppm; ABX system: δ_A = 2.775/2.784, δ_B = 2.94/2.95, δ_X = 4.35 (J_{AB} = 13.6, J_{AX} = 8.4, J_{BX} = 5.1) ppm; δ = 7.27 (br. s, 4 H, OH, COOH, NH_2), 8.20 (d, J = 8.1 Hz, 1 H, NH) ppm. ^{13}C NMR (75.5 MHz, [D_6]DMSO, 2 diastereomers): δ = 22.4 (Ac), 33.8/33.9 (*C*H$_2$CHNH), ca. 36 (br., *C*D$_2$CHOH), 52.30/52.35 (C*H*NH), ca. 71 (br., CD*H*OH), 169.5, 172.2, 174.83/174.88 (CO) ppm. MS (ES): *m*/*z* (negative mode, %) = 505 (100) $[2\ M - H]^-$, 252 (39) $[M - H]^-$.

***N*-Acetyl-*S*-[(*R*/*S*)-1-carbamoyl-2-hydroxyethyl]cysteine (5-H) and *N*-Acetyl-*S*-{(*R*/*S*)-1-carbamoyl-2-hydroxy[1,2,2-D$_3$]ethyl}cysteine (5-D):** Compounds **5**-H and **5**-D were prepared from the esters **10**-H (32 mg, 0.10 mmol) and **10**-D (100 mg, 0.33 mmol) in water (0.5 and 1 mL, respectively) with concd. aq. HCl (0.2 and 0.3 mL, respectively), as described above for the major regioisomer **4**-H. The products were isolated by preparative HPLC as glassy oils; t_R = 4.5/4.7 min (2:3, 2 diastereomers); 11 mg (42%) of **5**-H, 36 mg (44%) of **5**-D. **5-H:** ^1H NMR (300 MHz, D_2O, major diastereomer): δ = 2.05 (s, 3 H) ppm; ABX system (SC*H*$_2$C*H*NH): δ_A = 3.02, δ_B = 3.19, δ_X = 4.63 (J_{AB} = 14, J_{AX} = 8.5, J_{BX} = 4.8) ppm; ABX system (OHC*H*$_2$C*H*S): δ_A = 3.80, δ_B = 3.84, δ_X = 3.57 (J_{AB} = 12, J_{AX} = 5.9, J_{BX} = 7.7) ppm. ^{13}C NMR (75.5 MHz, D_2O, 2:1 mixture of 2 diastereomers): δ = 24.85 (Ac, assigned chemical shift), 35.4/35.5 (CH_2S), 53.4/53.6 (CHS), 55.6/55.8 (CHN), 64.9 (CH_2O), 176.6, 177.46/177.43, 178.7/178.8 (CO) ppm. HRMS ($C_8H_{14}N_2O_5S$): *m*/*z* (ESI, positive mode; %) = 273.05169 (100) [M + Na] {273.05156 (calcd.)}, 251.06977 (20) [M + H] {251.06962 (calcd.)}. **5-D:** ^1H NMR (300 MHz, D_2O, 2:1 mixture of diastereomers): δ = 2.04 (s, 3 H)ppm; ABX system: δ_A = 3.00/3.03, δ_B = 3.17, δ_X = 4.62 (J_{AB} = 14, J_{AX} = 8.3, J_{BX} = 4.7) ppm. ^{13}C NMR (75.5 MHz, D_2O, 2:1 mixture of diastereomers): δ = 24.85 (Ac, assigned chemical shift), 35.4/35.5 (CH_2S), ca. 53.2 (br., CD), 55.6/55.7 (CHN), ca. 64.1 (br., CD_2), 176.6, 177.42/177.38, 178.66/178.75 (CO) ppm. HRMS ($C_8H_{11}D_3N_2O_5S$): *m*/*z* (ESI, positive mode; %) = 276.07033 (100) [M + Na] {273.07040 (calcd.)}, 254.08839 (75) [M + H] {254.08845 (calcd.)}.

***N*-Acetyl-*S*-[(*R*/*S*)-2-cyano-2-hydroxyethyl]cysteine (12-H) and *N*-Acetyl-*S*-[(*R*/*S*)-1-cyano-2-hydroxyethyl]cysteine (13-H):** A suspension of *N*-acetylcysteine (**3**) (163 mg, 1.00 mmol) in water (3 mL) was flushed with argon, cooled to 4 °C, then 20% aq. Na_2CO_3 (0.5 mL) was added, and subsequently cyanooxirane **11** (140 mg, 2.03 mmol). The reaction mixture was warmed up to room temperature with rapid stirring, and after 1 h, it was acidified with 1 M aq. HCl to pH = 2. Then it was concentrated in vacuo to ca. 1 mL, and the title compounds were isolated by preparative RP-HPLC (column 8 × 250 mm; A/B, 96:4; 10 injections, 0.1 mL each): t_R = 6.6 min (for both epimers of the minor regioisomer **13**-H), yield 9 mg (4%) of a glassy solid; 8.2 min (for both epimers of the major regioisomer **12**-H), yield 85 mg (37%) of a glass-like foam. **12-H:** ^1H NMR (300 MHz, D_2O): δ = 2.08 (s, 3 H) ppm; ABX system (SC*H*$_2$C*H*OH): δ_A/δ_B = 3.03–3.07, δ_X = 4.84 ppm; ABX system (SC*H*$_2$C*H*NH): δ_A = 3.08, δ_B = 3.24, δ_X = 4.65 (J_{AB} = 14, J_{AX} = 8, J_{BX} = 5) ppm. ^{13}C NMR (75.5 MHz, D_2O): δ = 22.4 (Ac), 34.2 (*C*H$_2$CHNH), 36.7 (*C*H$_2$CHOH), 53. 3 (C*H*NH), 61.7 (C*H*OH), 120.6 (CN), 174.2, 174.9 (CO) ppm. MS (ES): *m*/*z* (negative mode, %) = 463 (100) $[2\ M - H]^-$, 231 (24) $[M - H]^-$. HRMS ($C_8H_{12}N_2O_4S$): *m*/*z* = 233.05922 [M + H] {233.05905 (calcd.)}, 255.04126 [M + Na] {255.04100 (calcd.)}.

***tert*-Butyl *N*-Acetyl-*S*-[(*R*/*S*)-2-(*tert*-butyldimethylsilyl)oxy-2-carbamoyl[1,1,2-D$_3$]ethylcysteinate (14):** Imidazole (122 mg, 1.82 mmol) and then *tert*-butyldimethylsilyl chloride (271 mg, 1.80 mmol) were added to a solution of hydroxyamide **9**-D

(473 mg, 1.53 mmol) in anhydrous DMF (2 mL). The reaction mixture was left at +5 °C for 4 d and was then taken up in an EtOAc/water mixture (10:1, 100 mL). The organic layer was separated, washed with water (20 mL), brine (20 mL) and dried. After evaporation of the solvent, the residual oil (652 mg, 100%) was used in the next step without further purification; R_f = 0.3 (EtOAc). ^{1}H NMR (300 MHz, $CDCl_3$, 1:1 mixture of 2 diastereomers): δ = 0.09/0.10/0.13/0.14 (s ×4, Me_2Si, Σ 6 H), 0.91 (s, 9 H, *t*BuSi), 1.44/1.45 (s, 9 H, *t*BuO), 2.00/2.03 (s, 3 H, ac), 2.89–3.10 (m, 2 H, SC*H*$_2$CHNH), 4.65–4.77 (m, 1 H, C*H*NH), 5.72/5.77 (br. s, 1 H, CON*H*H), 6.34/6.73 (d ×2, *J* = 7.8/7.9 Hz, Σ 1 H, N*H*CH), 6.59 (br. s, 1 H, CONH*H*) ppm. MS (ES): *m*/*z* (positive mode; %) = 869 (100) [2 M + Na]$^+$, 446 (13) [M + Na]$^+$; *m*/*z* (negative mode) = 468 (100) [M + HCOO$^-$].

***tert*-Butyl *N*-Acetyl-*S*-{(*R*/*S*)-2-(*tert*-butyldimethylsilyl)oxy-2-cyano[1,1,2-D$_3$]ethyl}cysteinate (15):** Pyridine (0.29 mL, 3.6 mmol) was added to a solution of amide **14** (652 mg, 1.54 mmol) in anhydrous CH_2Cl_2 (5 mL) at 0 °C, and subsequently trifluoroacetic anhydride (0.21 mL, 1.8 mmol) was added dropwise with stirring. The reaction mixture was stirred at room temperature overnight, diluted with CH_2Cl_2 (50 mL), washed with water (20 mL), 0.5 M aq. H_2SO_4 (20 mL), water (20 mL) and brine (20 mL). After drying and evaporation of the solvent, the residue was subjected to column chromatography on SiO_2 (100 mL) eluting with a hexane/EtOAc (1:1) mixture, to yield the title compound as a colorless oil (415 mg, 66%); R_f = 0.8 (EtOAc), R_f = 0.3 (hexane/EtOAc, 1:1). ^{1}H NMR (300 MHz, $CDCl_3$, 1:1 mixture of 2 diastereomers): δ = 0.13/0.14/0.17/0.18 (s ×4, Me_2Si, Σ 6 H), 0.879/0.885 (s, 9 H, *t*BuSi), 1.46 (s, 9 H, *t*BuO), 2.01/2.02 (s, 3 H, ac), 2.97–3.14 (m, 2 H, SC*H*$_2$CHNH), 4.66 (m$_c$, 1 H, C*H*NH), 6.32 (d, *J* = 7.3 Hz, 1 H, N*H*CH) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$, mixture of 2 diastereomers): δ = –5.31/–5.25 (Me_2Si), 18.0 (C), 23.1 (Ac), 25.4 (Me), 27.9 (Me), 35.4/35.5 (*C*H$_2$S), ca. 37.5 (CD_2), 52.5/52.6 (C*H*NH), ca. 62.3 (CD), 83.20/83.23 (CO), 118.98/119.00 (CN), 169.50/169.52, 169.79/169.83 (CO) ppm. MS (ES): *m*/*z* (positive mode; %) = 833 (49) [2 M + Na]$^+$, 428 (100) [M + Na]$^+$; *m*/*z* (negative mode; %) = 450 (100) [M + HCOO$^-$], 464 (62) [M + AcO$^-$].

***N*-Acetyl-*S*-{(*R*/*S*)-2-cyano-2-hydroxy[1,1,2-D$_3$]ethyl}cysteine (12-D):** Et_3SiH (0.15 mL, 0.11 g, 0.94 mmol) was added to a solution of the nitrile **15**-D (61 mg, 0.15 mmol) in CH_2Cl_2 (0.3 mL) at 0 °C, and subsequently TFA (0.18 mL, 2.34 mmol) was added. The reaction mixture was kept at +5 °C for 2 d and at room temperature for 1 d, concentrated in vacuo and taken up with $CHCl_3$ (2 × 10 mL), then concentrated again. The residue was dissolved in MeCN (0.15 mL), and aq. HF (51–56%, 0.1 mL) was added at 0 °C. After stirring at room temperature for 2 h, oily drops of *t*BuMe_2SiF appeared in the reaction mixture. It was concentrated in vacuo, taken up with water (2 × 1 mL), concentrated again and the residue subjected to preparative HPLC (A/B, 94:6) to yield the title compound (t_R = 8.2 min) as a viscous oil (14 mg, 40%). ^{1}H NMR (300 MHz, D_2O): δ = 2.13 (s, 3 H) ppm; ABX system (SC*H*$_2$C*H*N*H*): δ_A = 3.14, δ_B = 3.30, δ_X = 4.69 (J_{AB} = 14, J_{AX} = 8, J_{BX} = 5) ppm. ^{13}C NMR (75.5 MHz, D_2O): δ = 22.7 (Ac), 34.4 (*C*H$_2$CHNH), ca. 36.5 (*C*D$_2$CHOH), 53.7 (C*H*NH), ca. 62 (C*D*OH), 120.9 (CN), 174.7, 175.3 (CO) ppm. MS (ES): *m*/*z* (negative mode; %) = 469 (100) [2 M – H]$^-$, 234 (23) [M – H]$^-$. HRMS ($C_8H_9D_3N_2O_4S$): *m*/*z* = 236.07797 [M + H] {236.07788 (calcd.)}, 258.05996 [M + Na] {258.05982 (calcd.)}, 234.06339 [M – H] {234.06333 (calcd.)}.

3-(*tert*-Butyldimethylsilyloxy)-2-hydroxypropanenitrile (20-H): In a suspension of the aldehyde **19**-H[21] (806 mg, 4.62 mmol) and $Na_2S_2O_5$ (977 mg, 5.14 mmol) in water (10 mL) and methanol (5 mL) kept at 0 °C in the presence of methanol (5 mL) appeared a colorless paste, and after 20 min, a solution of KCN (0.585 g, 9.0 mmol) in water (1.5 mL) was added dropwise at 0 °C. ***CAUTION:** The reaction should be carried out in a well-ventilated hood, and the remaining KCN in all aqueous solutions after the workup should be oxidized with $KMnO_4$ before disposal!* The reaction mixture was left to warm to room temperature and stirred for 2 h. Water (20 mL) and subsequently diethyl ether (50 mL) were added. The organic layer was separated, washed with brine, dried and concentrated in vacuo. The residue (0.75 g, corresponding to 81% yield of the title compound) was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.11/0.12 (s ×2, 6 H, Me_2Si), 0.91 (s, 9 H, *t*Bu), 3.05 (d, *J* = 8.6 Hz, 1 H, OH), 3.84 (d, *J* = 3.8 Hz, CH_2O), 4.46 (m$_c$, 1 H, CHCN) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = –5.5 (Me_2Si), 18.2 (C), 25.7 (Me_3C), 62.2 (CHCN), 64.2 (CH_2), 118.3 (CN) ppm. MS (CI, NH_3): *m*/*z* = 219 [M + NH_4^+].

3-(*tert*-Butyldimethylsilyloxy)-2-hydroxy[2,3,3-D$_3$]propanenitrile (20-D): Compound **20**-D was synthesized from [D$_4$]ethylene glycol (OH,OD) (5 g) which was monoprotected with *tert*-butyldimethylsilyl chloride as described for the unlabelled compound,[20] and the alcohol **18**-D was then oxidized to the aldehyde **19**-D according to the procedure described previously for the unlabelled compound.[21] The reaction of **19**-D with aq. KCN in the presence of $Na_2S_2O_5$ was carried out as described above for compound **20**-H. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.10/0.11 (s ×2, 6 H, Me_2Si), 0.90 (s, 9 H, *t*Bu), 3.20 (br. s, 1 H, OH) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = –5.5 (Me_2Si), 18.3 (C), 25.7 (Me_3C), ca. 62 (t, CDCN), ca. 64 (m, CD_2), 118.5 (CN) ppm.

3-(*tert*-Butyldimethylsilyloxy)-2-(tosyloxy)propanenitrile (21-H): To a solution of the crude cyanohydrine **20**-H (4.2 g, 21 mmol) in pyridine (12 mL) was added tosyl chloride (4.2 g, 22 mmol) at 0 °C. The mixture was left at +5 °C overnight, poured into a cold mixture of concd. HCl (12 mL) and ice (50 g), and then $CHCl_3$ (50 mL) was added. The mixture was shaken, the organic layer was separated, and the aqueous layer was extracted with $CHCl_3$ (2 × 30 mL). The combined organic layers were washed with a phosphate buffer solution (pH = 7, 100 mL), dried and concentrated in vacuo. The title compound **21**-H (6.5 g, 83%) was isolated as a clear colorless oil by column chromatography on SiO_2 (100 g) eluting with a hexane/ether mixture (5:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.06 (s, 6 H, Me_2Si), 0.85 (s, 9 H, *t*Bu), 2.45 (s, 3 H, Me), 3.91 (d, *J* = 5.8 Hz, 1 H, CH_2O), 5.02 (t, *J* = 5.8 Hz, CHCN), 7.37 (d, *J* = 8.2 Hz, 2 H, H^{ar}), 7.82 (d, *J* = 8.4 Hz, 2 H, H^{ar}) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = –5.6/–5.6 (Me_2Si), 18.1 (C), 21.7 (Me), 25.5 (Me_3C), 63.0 (CH_2O), 67.0 (CHCN), 114.0 (CN), 128.2 (CH), 130.2 (CH), 131.9 (C), 146.2 (C) ppm. MS (CI, NH_3): *m*/*z* (%) = 728 (96) [2 M + NH_4^+], 373 (100) [M + NH_4^+].

3-(*tert*-Butyldimethylsilyloxy)-2-tosyloxy[2,3,3-D$_3$]propanenitrile-(21-D): Compound **21**-D was obtained from **20**-D (1.33 g, 6.5 mmol) in pyridine (5 mL) with *p*-toluenesulfonyl chloride (1.33 g, 7.0 mmol) in 92% yield (2.15 g) as described above for compound **21**-H. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.06 (s, 6 H, Me_2Si), 0.85 (s, 9 H, *t*Bu), 2.45 (s, 3 H, Me), 7.37 (d, *J* = 8.2 Hz, 2 H, H^{ar}), 7.82 (d, *J* = 8.4 Hz, 2 H, H^{ar}) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = –5.6/–5.6 (Me_2Si), 18.1 (C), 21.7 (Me), 25.7 (Me_3C), 62.5 (m, CD_2O), 66.8 (t, CDCN), 114.0 (CN), 128.2 (CH), 130.2 (CH), 132.1 (C), 146.1 (C) ppm. MS (CI, NH_3): *m*/*z* (%) = 376 (100) [M + NH_4^+].

***N*-Acetyl-*S*-[(*R*/*S*)-1-cyano-2-hydroxyethyl]cysteine (13-H ≡ 23a):** Et_3N (1 mL) was added to a solution of tosylate **21**-H (0.33 g, 0.92 mmol) and *N*-acetylcystein (0.21 g, 1.3 mmol) in MeOH

(5 mL) under Ar, and the mixture was stirred at 40 °C for 36 h. The solvent and excess of base were evaporated in vacuo, and satd. aq. $NaHCO_3$ (10 mL) was added to the residue. The mixture was extracted with diethyl ether (3 × 20 mL), and the organic layers were discarded. The aqueous layer was acidified with 5% aq. citric acid (pH ≈ 3) and then extracted with diethyl ether (3 × 20 mL). The combined organic solutions were dried and the solvents evaporated in vacuo. The residue (ca. 0.1 g of the crude compound **22a**) was dissolved in THF (2 mL), and a cold mixture of concd. HCl (0.5 mL) and water (0.2 mL) was added carefully at 0 °C. After having kept the mixture at 0 °C for 2 h, it was concentrated in vacuo (ca. 1 mbar) at 0 °C, the residue was dissolved in water (1 mL), and the title compound was isolated by preparative RP-HPLC (column 8 × 250 mm; A/B, 96:4; 10 injections, 0.1 mL each): t_R = 6.6 min (for both epimers of the regioisomer **13**-H), yield 30 mg (14%) of a glassy solid (after 3 co-evaporations with water to remove traces of TFA from the eluent and lyophilization). ^{1}H NMR (300 MHz, D_2O, mixture of 2 diastereomers): δ = 2.14 (s, 3 H) ppm; ABX system (cysteine residue): δ_A = 3.25/3.27, δ_B = 3.42/3.44, δ_X = 4.79 (J_{AB} = 14.2, J_{AX} = 7.7/8.3, J_{BX} = 4.8/5.3) ppm; ABX system [SC*H*(CN)C*H*$_2$OH]: δ_A = 3.94, δ_B = 4.02, δ_X = 4.13 (t) (J_{AB} = 11.4, J_{AX} = 6.2, J_{BX} = 5.2) ppm. ^{13}C NMR (75.5 MHz, D_2O): δ = 22.39/22.42 (Ac), 33.28/33.40 (*C*H$_2$S), 36.35/36.46 (*C*HCN), 52.96/53.12 (C*H*NH), 62.15/62.12 (C*H*$_2$OH), 119.69/119.72 (CN), 173.88/173.91, 174.88/174.94 (CO) ppm. MS (ESI): *m*/*z* (negative mode, %) = 463 (100) [2 M – H]$^-$, 231 (24) [M – H]$^-$. HRMS ($C_8H_{12}N_2O_4S$): *m*/*z* (%) = 233.05908 (60) [M + H] {233.05905 (calcd.)}, 255.04105 (100) [M + Na] {255.04100 (calcd.)}, 271.00606 (37) [M + K].

***N*-Acetyl-*S*-{(*R*/*S*)-1-cyano-2-hydroxy[2,2-D$_2$]ethyl}cysteine (23b):** Compound **23b** was obtained from the tosylate **21**-D as described above for compound **23a**. ^{1}H NMR (300 MHz, D_2O, mixture of 2 diastereomers): δ = 2.14 (s, 3 H) ppm; ABX system (cysteine residue): δ_A = 3.22/3.26, δ_B = 3.39/3.43, δ_X = 4.76/4.78 (J_{AB} = 14, J_{AX} = 7.8/8.3, J_{BX} = 4.65/5.05) ppm; 4.10 [s, 1 H, SC*H*(CN)CD_2OH] ppm. ^{13}C NMR (75.5 MHz, D_2O): δ = 21.8 (Ac), 32.66/32.78 (*C*H$_2$S), 35.57/35.69 (*C*HCN), 52.37/52.532 (C*H*NH), ca. 61.5 (CD_2), 119.08 (CN), 173.31/173.34, 174.30/174.36 (CO) ppm. HRMS ($C_8H_{10}D_2N_2O_4S$): *m*/*z* (%) = 235.07171 (100) [M + H] {235.07160 (calcd.)}, 257.05370 (49) [M + Na] {257.05355 (calcd.)}.

***N*-Acetyl-*S*-{(*R*/*S*)-1-cyano-2-hydroxy[1,2,2-D$_3$]ethyl}cysteine (23c):** Compound **23c** was obtained from the tosylate **21**-D (0.36 g, 1.0 mmol) and *N*-acetylcystein (0.28 g, 1.7 mmol) in MeOD (10 mL) with Et_3N as described above for compound **23a** (≡ **13**-H). The residue after evaporation of the solvent and the excess of Et_3N, was treated with D_2O (6 mL) and Na_2CO_3, until the pH value of the aqueous phase reached 9–10. The mixture was extracted with diethyl ether (3 × 5 mL), and the aqueous layer was carefully acidified at 0 °C with D_2SO_4 diluted with D_2O (1:4), until the pH value of the solution reached 3. Further workup was performed as described above for compound **23a**, and finally 32 mg (14%) of the title compound **23c** was isolated. ^{1}H NMR (300 MHz, D_2O, mixture of 2 diastereomers): δ = 2.13 (s, 3 H) ppm; ABX system (cysteine residue): δ_A = 3.23/3.27, δ_B = 3.38/3.42, δ_X = 4.77/4.80 (J_{AB} = 14.1, J_{AX} = 7.6/8.2, J_{BX} = 4.6/5.1) ppm. ^{13}C NMR (75.5 MHz, D_2O): δ = 22.35/22.39 (Ac), 33.2/33.3 (*C*H$_2$S), ca. 36 (m, *C*DCN), 52.9/53.1 (C*H*NH), ca. 62 (m, CD_2), 119.7 (CN), 173.82/173.85, 174.89/174.95 (CO) ppm. MS (ESI): *m*/*z* (negative mode; %) = 469 (100) [2 M – H]$^-$, 234 (16) [M – H]$^-$. HRMS ($C_8H_9D_3N_2O_4S$): *m*/*z* (%) = 236.07792 (100) [M + H] {236.07788 (calcd.)}, 258.05992 (63) [M + Na] {258.06038 (calcd.)}.

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[23] The use of $[D_6]$acetone allows one to identify the signals of the CH_2OH group: in our case the OH proton gave a broad signal at $\delta \approx 5.0$ ppm, and the CH_2 group was detected as a broad multiplet at $\delta = 3.83$ ppm; Linhart found the former signal for the two diastereomers at $\delta = 4.84$ and 4.87 ppm as two triplets with $J = 6.2$ Hz and the latter at $\delta = 3.85$ ppm as a multiplet. The chemical shifts of the CHS(CN) fragment signals were found to be the same as reported in ref.[15]: $\delta = 4.01$ and 4.05 ppm ($J = 5.4$ and 6.9 Hz) for 2 diastereomers.

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