Synthesis of Mixed Disulfides with Cyanogen Bromide and Its Consequences for Elucidation of Protein Structure

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For a study of the mechanism of a new bacterial enzyme that catalyzes the synthesis of γ-cyanoaminobutyric acid and thiocyanate from a mixture of homocystine (1) and cyanide, a route to γ-thiocyano-α-aminobutyric acid (3) as a possible intermediate was sought.[1] For this purpose homocysteine (4) was treated with cyanogen bromide. During the course of the alkylation, which furnished the desired thiocyanoamino acid in low yield, large amounts of homocystine also formed. This suggestion, that CNBr was acting in effect also as a thiol oxidizing agent, became even clearer when an attempt was made to prepare the starting 4 *in situ* by reduction of the more available 1 with 10 equiv of 2-mercaptoethanol before treatment with CNBr. After 2 hr at pH 9.3 the mixture contained, as determined on the amino acid analyzer,[2] 43% 4, 7% 1, and 39% of the new mixed disulfide of homocysteine and 2-mercaptoethanol, 1-DL-amino-7-hydroxy-4,5-dithiaheptane-1-carboxylic acid (6). Addition of 12.5 equiv of CNBr resulted in a mixture of 9% 4, 12% 1, and 72% 6. Apparently CNBr served to combine 1 equiv of 2-mercaptoethanol with 1 equiv of homocysteine liberated by nucleophilic attack of 2-mercaptoethanol on homocystine, to give a second mole of the mixed disulfide, 6. The effective reaction is the conversion of both alkyl residues of a symmetrical dialkyl disulfide into 2 mol of a mixed disulfide. In this respect it is similar to the excellent method of Schöberl and Gräfje for mixed disulfides,[3] in which the symmetrical disulfide is oxidized first with peracid to a thiolsulfinate ester that is then treated with a mercaptan. When applicable the combined reduction-CNBr procedure may be more convenient than the thiolsulfinate route. Formation of a symmetrical disulfide as a by-product in the synthesis of alkyl thiocyanates from mercaptans and CNBr has been noticed occasionally by others.[4,5] Moreover, in work on antiradiation compounds, Foye and coworkers reported an unexpected synthesis of 2-aminoethyl disulfide and 2-guanidinoethyl disulfide from the mercaptans and CNBr which they stated was applicable to a number of other symmetrical disulfides, including cystine and homocystine.[6]

DL-Homocysteine-2-mercaptoethanol mixed disulfide, 6, the product of sequence 1, was desalted and purified on a small Dowex 50-X8 resin column and, after crystallization, was obtained in 44% yield. Treatment of the sulfhydryl compound L-4 with excess 2-mercaptoethanol and CNBr furnished 6a in somewhat higher initial yield and purity (88% 6a and 12% 1) than the route starting from the disulfide 1. The product was isolated directly from the reaction mixture by crystallization. Like homocystine and γ-thiocyanoaminobutyric acid (3), 6a is a substrate for the enzymatic synthesis of γ-cyanoaminobutyric acid, presumably by first undergoing cyanolysis to 3.[1]

L-Cysteine (5) with excess 2-mercaptoethanol and CNBr formed in 93% yield the corresponding mixed disulfide of 5 and 2-mercaptoethanol, 1-L-amino-6-hydroxy-3,4-dithiahexane-1-carboxylic acid (7), that was also isolated directly by crystallization. It was chromatographically homogeneous and had the expected nmr spectrum. Its elution volume on the analyzer between proline and glycine was consistent with that reported previously for this compound when tentatively identified in an acid hydrolysate of mercaptoethanol-treated β-mercaptopyruvate transsulfurase.[7] Discrepant constants for 7 appear in the literature. The CNBr product agreed in melting point with 7 prepared by cooxidation of 5 and 2-mercaptoethanol[8] and in optical rotation with 7 prepared by treatment of 2-mercaptoethanol disulfide with 5.[3] The high isolated yields of 6a and 7 are attributed in part to the use of the large excess of 2-mercaptoethanol and to the ready removal by crystallization of the by-product, presumably 2-mercaptoethanol disulfide.

L-Homocysteine-2-mercaptoacetic acid mixed disulfide, 1-L-amino-4,5-dithiahexane-1,6-dicarboxylic acid (8), was also required for the study of the mechanism of the enzymatic utilization of homocystine.[1] It was prepared in a similar way from 4, excess 2-mercaptoacetic acid, and CNBr. DL-8 had been synthesized in 30% yield by reaction of DL-homocystine thiolsulfinate with 2-mercaptoacetic acid.[3]

Treatment of an equimolar mixture of L-homocysteine and L-cysteine with 2 equiv of CNBr in 0.1 *N* HCl gave, along with small amounts of 1 and 2, 59% of L-homocysteine–cysteine mixed disulfide 1-L,7-L-diamino-4,5-dithiaheptane-1,7-dicarboxylic acid (9). Identification of the latter was based on its chromatographic appearance on the analyzer in the "leucine–isoleucine area" previously described for this compound.[9,10] A chromatographic procedure is available for purifying 9 from a similar mixture also containing 1 and 2.[10] This mixed disulfide, which is excreted by individuals with cystinuria and homocystinuria[9,11] and with a derangement of B_{12} metabolism,[12] had been synthesized before in undisclosed yield by cooxidation in air of a mixture of 4 and 5.[9,10] DL-Homocysteine–L-cysteine mixed disulfide had been synthesized in 60% yield by reaction of DL-homocystine thiolsulfinate with 5.[3]

Symmetrical disulfides were prepared by treating 0.29 *M* mercaptan in 0.1 *N* HCl with approximately 2 equiv of CNBr. L-Cysteine yielded L-cystine (2); after one reprecipitation this product, obtained in 85% yield, had an optical rotation that agreed within 2% with the reported value for 2. 4 yielded 55% 1 and 8% 3; glutathione yielded 91% oxidized glutathione (10) as determined on the amino acid analyzer. CNBr should therefore be added to the group of mild oxidizing agents including O_2, H_2O_2, I_2, CH_2I_2, and

$K_3Fe(CN)_6$ that can be useful for synthesizing mixed and symmetrical disulfides from mercaptans.[13]

CNBr has come into extensive use for elucidation of protein structure for its specific degradation at methionine residues.[14] However, the significance of the disulfide-forming side reaction in this connection seems largely to have gone unnoticed. The early reports of Gross and Witkop on the CNBr degradation of proteins mention only that cysteine reacts slowly at its sulfhydryl group with CNBr[14] and that it is oxidized slowly to cysteic acid.[15] It was suggested that a sulfhydryl-containing protein or peptide be converted to its *S*-carboxymethyl derivative prior to treatment with CNBr,[15] a procedure that should circumvent disulfide formation as well as oxidation of cysteinyl to cysteyl residues. Although this suggestion has generally been followed, *e.g.*, an investigation of the half-cystine containing calf thymus F_3 histone,[16] some cysteine-containing proteins have been treated with CNBr without prior protection and the products have been examined directly. These include the monomeric component of hemoglobin from the bloodworm, *Glycera dibranchiata*,[17] and human serum albumin.[18] The latter, which has a single sulfhydryl group, yielded similar fragment resolution whether or not it had been pretreated with iodoacetate before being subjected to CNBr. However, with the unprotected albumin, aggregation took place and was attributed to incomplete cleavage and disulfide interchange.[18] When myosin, with about 42 sulfhydryl groups,[19] was treated in preliminary studies with CNBr at pH 3 and 6.5, extensive precipitation and gel formation took place, and a soluble macromolecular product could not be isolated.[20] It is clear that disulfide formation could be a factor in the aggregation process for cysteine-containing proteins when treated with CNBr. Such disulfide products would constitute erroneous structures. It has sometimes been the practice to treat an unprotected protein with CNBr and, to obtain still smaller fragments, reduce with dithiothreitol or 2-mercaptoethanol and then to *S*-carboxymethylate. Although the reductive procedure would tend to dissipate disulfides formed by CNBr, to complete the structure elucidation it may be necessary to study unreduced overlap peptides, and interpretation errors due to CNBr-mediated disulfide formation could arise at this point. The advisability of suitably modifying protein sulfhydryl before treatment with CNBr is again evident.

Experimental Section

DL-Homocystine and cysteine·HCl·H_2O were from Mann Research Laboratories, 2-mercaptoethanol (ME) was from Sigma, and oxidized glutathione was from C. F. Boehringer and Sons. Glutathione and cyanogen bromide (99.8% as redetermined by I_2–thiosulfate titrimetry)[21] were from Matheson Coleman and Bell. L-Homocysteine was prepared as described.[22] Thin layer chromatography (tlc) was on strips of Eastman Chromagram 6064 cellulose, and paper chromatography (pc) was on Whatman No. 1 in pyridine–water–*n*-butyl alcohol–acetic acid (1:2:4:1). The strips were treated with ninhydrin.

The nmr spectrum was determined at 60 MHz with a Varian Model EM-360 nmr spectrometer. Chemical shifts are expressed in δ values (parts per million) relative to a Me_4Si external standard. Melting points and optical rotations were taken and amino acid analyses with systems A and B were carried out as described elsewhere.[23] System C refers to system B but at 15°. Elution volumes and ninhydrin color-yield constants were as follows: in system A, **10**, 231 ml, *c* 31.6; **7**, 280 ml between proline and glycine,[7] *c* 15.9; **9**, 517 ml (45 ml after indication of the change to 4.26 and 50°),[23] *c* 37 (the constant for homolanthionine was assumed; **8**, 5 ml before **1** and 9 ml before Met, *c* 21; in system C, **4**, 48 ml, *c* 21; **1**, 109 ml, *c* 37.6; **6**, 93 ml, *c* 20.3. **1** and **6** did not separate in system B. Reactions were generally under N_2 and were followed by the disappearance of sulfhydryl as determined by the nitroprusside test on paper.[24]

DL-Homocysteine-2-Mercaptoethanol Mixed Disulfide [1-DL-Amino-7-hydroxy-4,5-dithiaheptane-1-carboxylic Acid (6)]. A suspension of DL-**1** (1.34 g, 5 mmol) in 200 ml of deaerated water was adjusted to pH 9 with concentrated NH_3 and stirred under N_2 with ME (3.49 ml, 50 mmol). After 2 hr it was readjusted to pH 9. To the clear solution CNBr (6.47 g, 61 mmol) dissolved in 50 ml of water was added in portions over 10 min at 25°. The solution was taken to dryness and the solid residue, 4.4 g, was collected with the aid of ethanol–ether. This was dissolved in 7 ml of hot water, acidified to pH 2, and applied to a Dowex 50-X8 (H^+) resin column (1.5 × 25 cm). The column was treated with water until the effluent became neutral and then with pyridine acetate buffer, pH 4.45. Fractions of 4 ml were collected. Elution progress was monitored by tlc. Fractions 33–40, R_f 0.52, were combined and lyophilized and yielded 1.28 g of **6**. Subsequent fractions contained **1**, R_f 0.2, as well as **6**. The product was crystallized from 8 ml of 70% ethanol and was recrystallized: 864 mg (41%) of lustrous plates, mp 216–217.5° dec, homogeneous on amino acid analysis and tlc.

Anal. Calcd for $C_6H_{13}NO_3S_2$ (211.3): C, 34.1; H, 6.0; N, 6.63; S, 30.4. Found: C, 34.1; H, 6.32; N, 6.74; S, 30.1.

L-Homocysteine-2-Mercaptoethanol Mixed Disulfide [1-L-Amino-7-hydroxy-4,5-dithiaheptane-1-carboxylic Acid (6a)]. A solution of L-**4** (1.08 g, 8 mmol) in 56 ml of 10% ME (80 mmol) in 0.1 *N* HCl was treated with CNBr (14 g, 133 mmol) as described for **7**. Crystallization from 70% ethanol yielded 765 mg (45%) of **6a** as lustrous needles, homogeneous on amino acid analysis and tlc, mp 229° dec, $[\alpha]^{24.5}D$ +42.6° (*c* 1, 1 *N* HCl).

Anal. Found: C, 34.2; H, 6.16; N, 6.71; S, 30.3.

L-Cysteine-2-Mercaptoethanol Mixed Disulfide [1-L-Amino-6-hydroxy-3,4-dithiahexane-1-carboxylic Acid (7)]. L-Cysteine·HCl·H_2O (1.05 g, 6 mmol) was dissolved in 42 ml of 10% ME (60 mmol) in 0.1 *N* HCl. To it a solution of CNBr (9.47 g, 90 mmol) in 60 ml of 0.1 *N* HCl was added portionwise with magnetic stirring and cooling to maintain room temperature. The mixture was then taken to dryness, 50 ml of water was added, and evaporation was repeated. The residue was taken up in water and adjusted to pH 5, and the solvent was removed. The residue was crystallized from 70% ethanol to give 887 mg, mp 188–189° dec.

Recrystallization yielded 807 mg (68%) of **7**, homogeneous on amino acid analysis: tlc, R_f 0.41; pc, R_f 0.35; mp 182.5–183.5° dec; $[\alpha]^{26}D$ −242° (*c* 1, 1 *N* acetic acid); $[\alpha]^{24}D$ −137° (*c* 0.5, 1 *N* HCl). The reported values are mp 161–162° dec; $[\alpha]^{20}D$ −140.8° (*c* 0.8, 1 *N* HCl);[3] and mp 184–187° dec; $[\alpha]^{20}D$ −92.1° (*c* 0.4);[8] nmr (NaOD) δ 2.95 (2 H, t, J = 6 Hz, $^5CH_2$), 3.28 (d, J = 4 Hz) and 3.11 (s, sum of 2 H, $^2CH_2$), 3.88 (2 H, d, J = 6 Hz, $^6CH_2$), 4.16 (1 H, t, J = 4 Hz, ^1CH.

Anal. Calcd for $C_5H_{11}NO_3S_2$ (197.3): C, 30.5; H, 5.62; N, 7.1; S, 32.5. Found: C, 30.2; H, 5.48; N, 7.23; S, 32.7.

L-Homocysteine-2-Mercaptoacetic Acid Mixed Disulfide [1-L-Amino-4,5-dithiahexane-1,6-dicarboxylic Acid (8)]. Prepared as described for **7** from L-**4**, 2-mercaptoacetic acid, and CNBr, **8** was obtained in a crude yield of 91%. It was isolated with Dowex 50-X8 resin as described for **6** and was recrystallized from 70% ethanol: 30% yield; mp 183–185°; $[\alpha]^{24}D$ +6.1° (*c* 0.7, H_2O).

Anal. Calcd for $C_6H_{11}NO_4S_2$ (225.3): C, 32.0; H, 4.92; N, 6.22; S, 28.5. Found: C, 32.0; H, 4.99; N, 6.28; S, 28.2

L-Cystine (2). A solution of L-cysteine·HCl·H_2O (2.63 g, 15 mmol) in 50 ml of 0.1 *N* HCl was treated with CNBr (3.71g, 35 mmol) in 50 ml of 0.1 *N* HCl as described for **7**. After evaporation the residue was dissolved in a minimum of water, and the solution was adjusted to pH 5 with concentrated NH_3 when the product precipitated. After being cooled this was filtered off, resuspended in 10 ml of water, dissolved by the addition of concentrated HCl, and reprecipitated in the same way. **2** was collected and washed with water until halide-free, then with ethanol and ether: yield 1.51 g (85%); $[\alpha]^{24.5}D$ −211° (*c* 1.1, 1 *N* HCl); reported for **2** $[\alpha]^{25}D$ − 215°.[25] It was homogeneous on amino acid analysis with a quantitative recovery.

L-Homocysteine-L-Cysteine Mixed Disulfide [1-L,7-L-Diamino-4,5-dithiaheptane-1,7-dicarboxylic Acid (9)]. A solution of **4** (13.8 mg, 0.1 mmol) and cysteine·HCl·H_2O (17.9 mg, 0.1 mmol) in 0.35 ml of 0.1 *N* HCl was treated with CNBr (30 mg, 0.28 mmol). After 20 min the solution was taken to dryness and the residue was taken up in water. Amino acid analysis showed 59% **8**, 15% **1**, and 13% **2**.

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Registry No. DL-1, 870-93-9; L-2, 56-89-3; L-4, 6027-13-0; L-5 HCl, 52-89-1; 6, 42855-17-4; 6a, 42855-18-5; 7, 38254-63-6; 8, 42855-20-9.

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The Lewis Acid Catalysis of Ene Reactions

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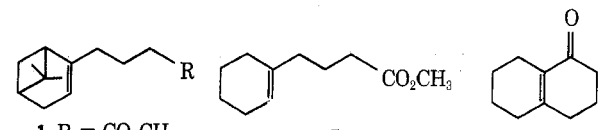
The ene reaction of olefins with highly activated enophiles has been well studied and is a synthetically useful reaction.[2] With less reactive enophiles such as methyl vinyl ketone and methyl acrylate, the ene reaction is of little value, since harsh conditions are required and yields are usually low.[2-4] Since Diels–Alder reactions often proceed in much higher yield and with greater stereospecificity in the presence of Lewis acid catalysts, it was decided to study the ene reaction of olefins with moderately reactive enophiles in the presence of these catalysts.

The reaction of (−)-β-pinene with methyl acrylate in the presence of aluminum chloride at *room temperature* gave the ene adduct **1** in 70% yield. Similarly, (−)-β-pinene with methyl vinyl ketone or acrolein in the presence of zinc bromide at *room temperature* afforded the adducts **2** and **3** in 62 and 32% yields, respectively. In the absence

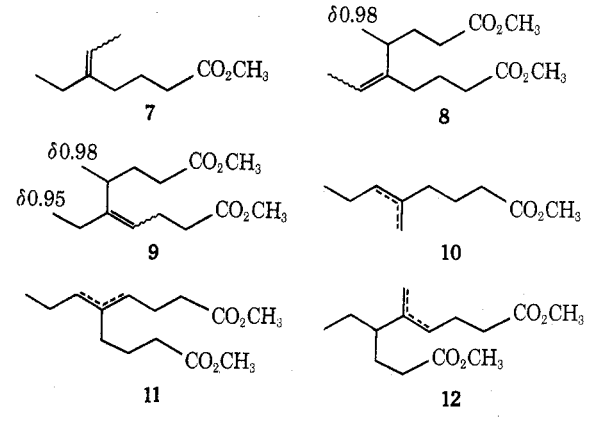


1, R = CO_2CH_3
2, R = $COCH_3$
3, R = CHO
4, R = CN

5 **6**

of Lewis acid catalysts, (−)-β-pinene reacted with acrolein at 135° for 17 hr to give a 30% yield of **3**[5] and with acrylonitrile at 230° for 6 hr to give a 41% yield of **4**.[4] From these data it is clear that Lewis acid catalysts greatly accelerate the ene reaction.

The reaction of methylenecyclohexane with methyl acrylate in the presence of aluminum chloride gave the known ester **5**[6] in 70% yield. The reported conversion of **5** to the acid chloride followed by a Friedel–Crafts type acylation results in a very short and efficient synthesis of the octalone **6** (59% from **5**).[6] The reaction of 2-ethyl-1-butene with methyl acrylate under similar conditions afforded a mixture from which the ene adduct **7** and the 2:1 adducts **8** and **9** were isolated in 59 and 9% yield, respectively. The 2:1 adducts are presumably obtained by ene reaction of the original adduct **7** with methyl acrylate. In support of this, it was found that, by allowing the reaction to proceed for a longer time in the presence of excess methyl acrylate, higher yields of **8** and **9** and lower yields of **7** were obtained. The 2:1 adduct mixture was identified by its mass spectra, by the doublet in the nmr spectra at δ 0.98 due to the methyl groups of **8** and **9**, and by the triplet at δ 0.95 due to the second methyl group of **9**. 2-Methyl-1-pentene and methyl acrylate under similar conditions gave a complex mixture of the 1:1 adducts **10** and the 2:1 adducts **11** and **12** which was not investigated further.



7 **8**

9 **10**

11 **12**

It has previously been observed that 1,1-disubstituted olefins give the highest yields in thermal ene reactions.[4] In this study ene reactions were obtained only with 1,1-disubstituted olefins. Preliminary experiments indicated that ene products were not obtained from methyl acrylate and allylbenzene, 1-octene, cyclohexene, or allyl bromide. It was also found that a mixture of products was obtained from (−)-β-pinene and methyl methacrylate or methyl *trans*-crotonate.

The ene reaction of olefins with dienophiles in the presence of Lewis acid catalysts provides an effective synthesis of compounds not readily available by other methods.

Experimental Section

Reaction of (−)-β-Pinene with Methyl Acrylate. To a solution of methyl acrylate (10.3 g, 0.12 mol) in 50 ml of dry benzene was added aluminum chloride (1.4 g, 0.01 mol). After the alumi-