809 s, 715 s; mass spectrum (75 eV) m/e (relative intensity) 66 (18), 91 (18), 115 (11), 117 (100), 118 (81), 184 (16).

X-Ray Diffraction. Needle crystals of XVb suitable for X-ray diffraction studies were obtained from methanol. The intensity data on which the refinement was carried out were collected on a needle (along b axis) with dimensions 0.07 × 0.05 mm and length 0.5 mm, mounted on a Picker FACS-1 diffractometer with Cu $K\alpha$ radiation and using the $\theta - 2\theta$ scan technique. The intensities were measured with a scintillation counter with background counts being made at each limit of the scan. A total of 2759 reflections was considered nonzero out to a 2θ limit of 130° using the criteria that the net count be greater than 0.06 times the total background count and/or 50 counts whichever is greater. No corrections were made for absorption. The range of transmission coefficients was calculated as 0.26–0.36.

Full-matrix least-squares refinement, varying positional and isotropic thermal parameters for the nonhydrogen atoms, gave values of R and R_2 ($R_2 = [\Sigma w(|F_o| - |F_c|)^2/\Sigma w|F_o|^2]^{1/2}$) of 0.182 and 0.166. All reflections were given unit weights and the quantity minimized was $\Sigma w(|F_o| - |F_c|)^2$. Least-squares refinement on positional and anisotropic thermal parameters for the nonhydrogen atoms reduced R and R_2 to 0.085 and 0.084. A difference map calculated at this point revealed several positive peaks in the range 0.2–0.4 electron/Å^3, some of which could represent hydrogen atoms. As the location of all of the hydrogen atoms could not be made with certainty, however, we decided to neglect the contribution of the hydrogen atoms and to terminate refinement.

The list of h, k, l, F_o and F_c values will appear in the microfilm edition.[29] The atomic scattering curves used for Br, C, and O were those tabulated by Cromer, *et al.*[44]

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Acetamidomethyl. A Novel Thiol Protecting Group for Cysteine

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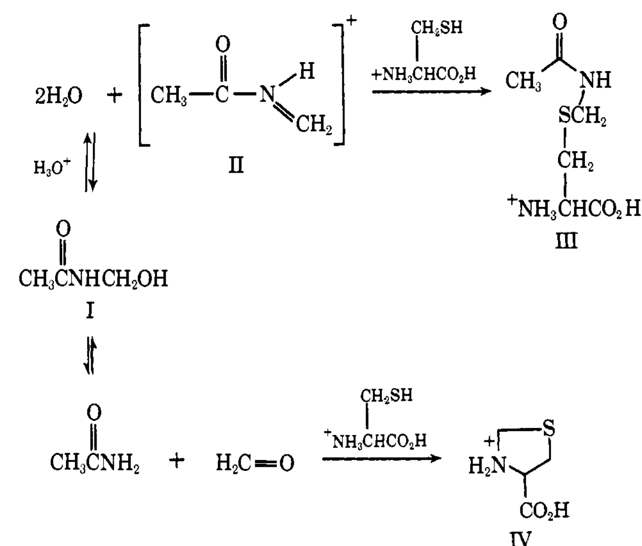
Abstract: The acetamidomethyl group has been found to be a useful protecting group for the thiol of cysteine. It was added to the thiol of cysteine and to the eight cysteines of the reduced S-protein of ribonuclease under acidic conditions. It is stable under the conditions commonly used in peptide synthesis and is removed by mercuric ion under mild conditions. The mercuric ion may be removed by treatment with H_2S for small peptides and by gel filtration in the presence of mercaptoethanol for large peptides and proteins.

In a preliminary communication[1] we have reported a novel derivative of cysteine in which the sulfur is protected by the acetamidomethyl (Acm) group.[2,3] This protecting group has been found to be useful both in the synthesis of peptides and proteins and in the reversible protection of a natural protein. In this report we describe in greater detail the preparation, properties, and utility of this derivative of cysteine.

Preparation of S-Acm-cysteine and Octa-S-Acm-octahydro-S-protein. S-Acm-Cys (III) was conveniently prepared in about 50% yield by treatment of cysteine with a 10% excess of N-hydroxymethylacetamide (I) in aqueous medium at pH 0.5. A minor byproduct, thiazolidine-2-carboxylic acid (IV), was removed by recrystallization. III was found to be stable at pH 0.5. We believe, therefore, that IV arose *via* the decomposition of I to formaldehyde and acetamide. Scheme I indicates that anhydrous conditions should shift the equilibrium between I and II to the right, and thus minimize the formation of IV.

Indeed, when cysteine was allowed to react with a 20% excess of I in anhydrous HF at 0°, III was formed in nearly quantitative yield, and IV was not detectable in the reaction mixture. More importantly, the use of liquid HF as solvent proved to be particularly useful when we applied it to the S-alkylation of reduced S-protein,[4] since acetamidomethylation of this protein in anhydrous but not in aqueous medium led to a product

Scheme I



(1) D. F. Veber, J. D. Milkowski, R. G. Denkewalter, and R. Hirschmann, *Tetrahedron Lett.*, 3057 (1968).

(2) A related class of protecting groups has been reported by F. Weygand, W. Steglich, I. Lengyel, F. Fraunberger, A. Maierhofer, and W. Oettmeier, *Chem. Ber.*, **99**, 1944 (1966).

(3) All amino acids are of the L configuration. Abbreviations used: Acm = acetamidomethyl; Boc = *tert*-butyloxycarbonyl.

(4) (a) F. Haber and C. B. Anfinsen, *J. Biol. Chem.*, **236**, 422 (1961); (b) S-protein is the protein obtained on treatment of ribonuclease-A with subtilisin (F. M. Richards, *Proc. Nat. Acad. Sci. U. S.*, **44**, 162 (1958)).

which could be reconverted to active enzyme. Treatment of octahydro-*S*-protein with a 20% excess of ^{14}C-labeled I in HF at 0° gave about 50% yield of a product which had nearly the same elution volume from Sephadex G-75 as *S*-protein and which had the specific radioactivity expected for octa-Acm-octahydro-*S*-protein. Amino acid analysis after enzymatic hydrolysis (Table II) indicated the presence of eight Acm-Cys residues and no cystine. Furthermore, the amino acid analyses after enzymatic and acid hydrolysis indicated that the other amino acids had been essentially unaffected by the alkylation. A similar alkylation of a mixture of all of the amino acids of *S*-protein but in the absence of cysteine showed that tyrosine also reacts with I. The other amino acids showed little or no tendency to react in the absence of the cysteine acceptor. The gel filtration also gave a by-product of higher molecular weight, probably a dimer. The yield of this by-product was variable (35–50%), and apparently is mainly dependent on the amount of oxygen present during the isolation of the octahydro-*S*-protein. The specific radioactivity of this dimer (or mixture of dimers) indicated the presence of only seven Acm groups. On reduction with mercaptoethanol and subsequent treatment with acetamidomethanol in HF, it was converted to octa-*S*-Acm-octahydro-*S*-protein. Thus, this by-product appears to arise as a result of oxidative dimerization *via* a disulfide bond leading to incomplete alkylation.

Stability Studies. The stability of the acetamidomethyl group of *S*-Acm-Cys and of Gly-*S*-Acm-Cys was studied under a wide variety of conditions including those commonly employed in peptide synthesis. These studies, summarized in Table I, indicate stability under

Table I. Stability of Acm-Cys and Gly-Acm-Cys

Reagent	Solvent	Temp, °C	Time, hr	Reaction,[a] %
pH 13	H_2O	25	5	None
1 *N* HCl	H_2O	25	24	None
6 *N* HCl	H_2O	110	20	100
1 *N* HCl	H_2O	100	0.25	Partial
15% NH_3	H_2O	25	24	None
Hydrazine (30 equiv)	HOAc	25	24	None
Hydrazine (30 equiv)	MeOH	25	2	None
Zn (dust)	50% HOAc–H_2O	25	24	None
Trifluoroacetic acid	Neat	25	5	None
6 *N* HCl	EtOAc	0	0.5	None
HF	Neat	0	1	None
HF	Neat	18	1	*Ca.* 10
30% HBr	HOAc	25	5	Partial

[a] Determined by tlc (ninhydrin spray; see Experimental Section).

a broad range of conditions. The protecting group is, in fact, nearly as stable as a peptide bond toward most reagents.

It was found to be hydrolyzed under the strongly acidic and strongly basic conditions commonly used for the hydrolysis of amide bonds. Thus, hydrolysis of peptides containing Acm-Cys at 110° in 6 *N* HCl for 20 hr, as commonly used prior to amino acid analysis, led to the formation of both cysteine and cystine. The relative quantities and total amount of the two amino acids were found to be dependent on the amount of care taken to exclude oxygen during hydrolysis and the nature of the other amino acids present in the peptide. Thus we found that tyrosine tended to prevent the oxidation of cysteine. Rigorous evacuation to remove oxygen prior to hydrolysis followed by an oxidation at pH 10 for 20 min prior to analysis gave high yields of cystine.

The oxidation of sulfides to sulfoxides is a well-recognized side reaction with methionine-containing peptides. This problem has only rarely been considered in studies of protected derivatives of cysteine.[5] We have found that *S*-Acm-Cys can be oxidized with hydrogen peroxide presumably to the diastereomeric sulfoxides, although III appears to be more stable toward air oxidation than methionine. The oxidation products were eluted at 36 min on the amino acid analyzer.[6,7] On reduction of the oxidation products with mercaptoethanol, *S*-Acm-Cys was obtained in quantitative yield. We believe that oxidation is not a problem with the Acm protecting group because we have never observed oxide formation during the normal course of a peptide synthesis using peroxide-free solvents and because oxides, if formed, could readily be reduced.

S-Acm-Cys reacts with strong electrophiles such as the *tert*-butyl cation which is generated during the removal of the *tert*-butyloxycarbonyl group. Thus, treatment of *S*-Acm-Cys with *tert*-butyl alcohol in trifluoroacetic acid led to about 1% S-alkylation in 40 min. This is about half the amount of alkylation observed with methionine under the same conditions. As expected, the alkylation of these amino acids could be suppressed completely by the addition of dimethyl sulfide.[8]

Since the acetamidomethyl protecting group is unstable to the usual conditions of acid hydrolysis required for amino acid analysis, the hydrolysis of a peptide must be carried out enzymatically in order to quantitatively determine the Acm-Cys content. Using leucineaminopeptidase or aminopeptidase-M to hydrolyze the peptide leaves the acetamidomethyl group intact. *S*-Acetamidomethylcysteine is eluted from the standard amino acid analysis columns[6,7] with aspartic acid and was found to have the same ninhydrin color constant. When a lithium buffer system[9] was used, *S*-Acm-Cys was eluted with threonine but was separable from aspartic acid. The combination of these two buffer systems thus permitted the quantitative determination of *S*-Acm-Cys in the presence of all the 20 coded amino acids and of cystine.

Racemization Studies. The active esters of cysteine derivatives used in peptide synthesis have been reported to be unusually prone to racemize[10] especially in the presence of a large excess of amine. We have extensively used Acm-Cys in peptide synthesis.[11,12] Neither

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(6) S. Moore, D. H. Spackmann, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
(7) Aspartic acid was eluted at 45 min.
(8) Unpublished observations, Dr. S. Brady of these laboratories.
(9) J. V. Benson, Jr., M. J. Gordon, and J. A. Patterson, *Anal. Biochem.*, **18**, 228 (1967).
(10) J. Kovacs, G. L. Mayers, R. H. Johnson, R. E. Cover, and U. R. Ghatak, *J. Org. Chem.*, **35**, 1810 (1970).
(11) R. Hirschmann, R. F. Nutt, D. F. Veber, R. A. Vitali, S. L. Varga, T. A. Jacob, F. W. Holly, and R. G. Denkewalter, *J. Amer. Chem. Soc.*, **91**, 507 (1969), and references cited therein.

tlc nor innumerable enzymatic degradations have given any indication of racemization of Acm-Cys.[12] Moreover, we determined the rate constant for the triethylamine-catalyzed racemization of (*N*-hydroxysuccinimido)-*N*-Cbz-*S*-Acm-cysteinate, and found it to be comparable to that of *N*-hydroxysuccinimido-*N*-Cbz-*S*-benzylcysteinate, reported by Kovacs, *et al.*[10] Thus racemization should not generally be a problem in the synthesis of Acm-protected peptides.

Removal of the Blocking Group. The acetamidomethyl function was removed from *S*-Acm-Cys and Gly-(*S*-Acm-Cys) by mercuric ion at pH 4 within 45 min. After removal of mercuric ion as HgS and subsequent air oxidation of the thiol, tlc showed cystine and diglycylcystine, respectively, to be the only ninhydrin positive products. The total yield of ninhydrin positive product was shown to be greater than 98%. In the preparation of diglycylcystine, nmr analysis of the total reaction mixture showed diglycylcystine and acetamidomethanol as the only detectable products. Thus, the removal of acetamidomethyl gave no observable by-products. Moreover, we have not seen any IV or related by-products which would arise from the addition of formaldehyde as has been reported for the removal of the benzylthiomethyl protecting group.[13] The removal of the acetamidomethyl function proceeded more slowly both above and below pH 4 (above pH 5 mercuric ion is precipitated as HgO). With peptides which are insoluble in water at pH 4, 50% aqueous acetic acid was found to be a satisfactory solvent for the removal of acetamidomethyl. The reaction was slower but was still complete in less than 2 hr. When required for solubility of the peptide, urea solutions (up to 8 *M*) have also proven to be satisfactory media for the removal of the S-blocking group. In the deprotection of octa-Acm-*S*-protein, optimal yields were obtained after treatment with 9 equiv of mercuric acetate for 70 min in 50% acetic acid. The removal of radioactivity from the octa-*S*-[^{14}C]-Acm-octahydro-*S*-protein described above was 98.8% under these conditions.

In the presence of peptides of low molecular weight, mercuric ion removal was most readily accomplished by addition of H_2S. Scrupulous removal of excess H_2S is required, however, to prevent the formation of *S*-sulfonates resulting from the oxidation of H_2S to SO_2. The H_2S method is not satisfactory with larger peptides which tend to be absorbed on mercuric sulfide. In these cases the method of choice for removal of Hg(II) involved use of excess mercaptoethanol and removal of the mercurimercaptoethanol by gel filtration. This latter method has been employed for the deprotection of *S*-acetamidomethylated octahydro-*S*-protein. Treatment with 9 equiv of mercuric acetate in 50% acetic acid for 70 min at 25° was followed by removal of mercuric ion as described above. The protein was air oxidized in the presence of *S*-peptide and purified by ion exchange chromatography. The enzymatically active material which had the same elution volume from a carboxymethyl Sephadex C-50 column as ribonuclease-*S* was found to have 90% the enzymatic activity of the native enzyme and could be crystallized by the procedure of Wyckoff, *et al.*,[14] on seeding with ribonuclease-*S*. The yield of product was about half that obtained from the oxidation of pure octahydro-*S*-protein. The reason for the reduction in yield is not known, but must be a result of by-products formed during the introduction and/or the removal of the Acm groups.

The usual procedures have been used to prepare Boc-*S*-Acm-cysteine,[15] Cbz-*S*-Acm-Cys and the *N*-hydroxysuccinimide esters[16] therefrom. These derivatives behave normally in peptide synthesis and have been used in the preparation of ribonuclease *S*-protein.[11] The use of these derivatives is exemplified herein with the synthesis of the ribonuclease fragment 82–85, Boc-Thr-Asp-Cys (Acm)-Arg-ethyl ester. Derivatives of *S*-Acm-Cys have also proven useful in the synthesis of peptides on a solid support.[17]

Discussion

Many protecting groups for the thiol function of cysteine have been described and reviewed.[18] Although each of these known protecting groups has many desirable properties, we feel that each one has some feature which makes it less than ideal as a cysteine protecting group.

We believe that the acetamidomethyl blocking group uniquely satisfies all of the following requirements. (1) It can be used for peptide synthesis either in solution or in the solid phase method. (2) It may be introduced and removed in high yield under mild conditions into cysteine-containing proteins such as reduced *S*-protein. (3) It is stable to both the mild acidic conditions required to remove, *e.g.*, the butyloxycarbonyl blocking group, and to treatment with liquid HF at 0° which affects removal of the benzyloxycarbonyl group. (4) It is stable also to conditions required to convert esters to hydrazides and hydrazides to azides, and to the weakly basic conditions employed in typical peptide coupling reactions. (5) The blocking group may be removed smoothly with Hg(II) under mild conditions. It is therefore possible to remove selectively either benzyloxycarbonyl or acetamidomethyl in a peptide containing both protecting groups.

Experimental Section

***S*-Acetamidomethyl-L-cysteine (III).** (a) A mixture of *N*-hydroxymethylacetamide[19] (127 g, 1.43 mol) and 228 g (1.3 mol) of cysteine hydrochloride monohydrate, dissolved in 350 ml of water, was cooled in an ice bath and 50 ml of concentrated hydrochloric acid was added (pH $\cong$ 0.5). The flask was stoppered and allowed tc stand 1–2 days at room temperature under nitrogen. When tlc (*n*-butyl alcohol–acetic acid–water (10:2:3), R_f 0.19, cysteine R_f 0.25) indicated completion of the reaction, the mixture was concentrated *in vacuo* at *ca.* 40° and absolute ethanol was added and evaporated several times to remove traces of water. The resulting solid was dissolved in a minimum of methanol at room temperature and anhydrous ether was added to the cloud point. The mixture was refrigerated several days, during which time the forming crystalline mass was broken up several times. The white crystalline solid was removed by filtration, washed with ether, and dried

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(13) P. J. E. Brownlee, M. E. Cox, B. O. Handford, J. C. Marsden, and G. T. Young, *J. Chem. Soc.*, 3832 (1964).

(14) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, D. Tsernoglou, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, **242**, 3749 (1967).

(15) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

(16) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964).

(17) Dr. George Gal of these laboratories, private communication.

(18) See for example: (a) E. Schröder and K. Lübke, "The Peptides," Vol. 1, Academic Press, New York, N. Y., 1965, p 226; (b) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience, New York, N. Y., 1966, p 54.

(19) A. Einhorn, *Justus Liebigs Ann. Chem.*, **343**, 207 (1905).

in vacuo to yield 158 g (52% yield; dec 159–163°; $[\alpha]^{25}_{589}$ −30.7° (*c* 1, H_2O)); the infrared spectrum showed carbonyl peaks at 5.82 and 6.25 μ (Nujol) and the nmr spectrum (D_2O–DCl) gave τ 7.95 (s, 3, CH_3), 6.80 (m, 2, CH_2), 5.80 (m, 1, CH), 5.61 (s, 2, CH_2).

Anal. Calcd for $C_6H_{13}N_2O_3SCl$: C, 31.51; H, 5.73; Cl, 15.50; N, 12.25; S, 14.02. Found: C, 31.19; H, 5.73; Cl, 15.62; N, 11.89; S, 14.30.

The free base was prepared by adding an equimolar amount of silver oxide to an aqueous solution of the hydrochloride, stirring the resulting mixture 15 min, filtering, and treating the filtrates with H_2S to precipitate Ag_2S. The product was recrystallized by dissolving in a minimum of hot H_2O and adding 2 vol of ethanol to yield the monohydrate, dec 187°; $[\alpha]^{25}_{589}$ −42.5° (*c* 1, H_2O).

Anal. Calcd for $C_6H_{12}N_2O_3S$ (dried at 100°): C, 37.48; H, 6.29; N, 14.57; S, 16.68. Found: C, 37.60; H, 6.48; N, 14.63; S, 16.76.

Some samples of *S*-Acm-Cys–HCl have been obtained which contain cystine–2HCl which is not easily removed by crystallization. Such samples were purified by conversion to the free base with NaOH. The Acm-Cys is considerably more soluble in H_2O than cystine and was crystallized on the addition of ethanol.

Thiazolidine-2-carboxylic acid is also found in small amounts (1–3%) during the reaction. A sample was isolated by silica gel chromatography and was shown to be identical with an authentic specimen by ir and nmr spectroscopy.

(b) A mixture of 450 mg (5.05 mmol) of hydroxymethylacetamide and 484 mg (4.0 mmol) of cysteine was dissolved in approximately 4 ml of anhydrous HF at −78° in a polyethylene test tube equipped with a drying tube. The mixture was then placed in an ice bath and was allowed to stand for 30 min with stirring. The bulk of HF was removed at 0° under a stream of N_2. The last traces of HF were removed in a vacuum dessicator over KOH. The residue was washed with 5 ml of ethanol and then with 5 ml of peroxide-free ether. Tlc analysis on silica gel G (butanol–acetic acid–water (10:2:3)) showed cystine as a minor impurity but no IV or other impurities.

The hydrofluoride salt was converted into the free base by dissolving the residue in 60 ml of H_2O and adjusting the pH to 5.3 with a saturated solution of $Ba(OH)_2$. The solution was allowed to stand for 0.5 hr and the precipitated BaF_2 was removed by filtration. The solution was evaporated to dryness and the residue was crystallized from H_2O–ethanol. The product was one component when analyzed by tlc; yield 577 mg (75%).

***N-tert*-Butoxycarbonyl-*S*-acetamidomethyl-L-cysteine.**[20] Acm-cysteine hydrochloride (68.4 g, 0.30 mol) was dissolved in 600 ml of dimethylformamide. Tetramethylguanidine (69 g, 0.6 mol) was added dropwise over 10 min while maintaining the reaction under a N_2 atmosphere and at a temperature of *ca.* 25° with a cold water bath. *tert*-Butoxycarbonyl azide (47.1 g, 0.33 mol) (Aldrich) was added dropwise over 10 min at 25° and an additional 34.5 g (0.3 mol) of tetramethylguanidine was added dropwise. The mixture was stirred overnight at room temperature. The reaction mixture was concentrated *in vacuo* at 30–35° and 200 ml of water was added. The aqueous mixture was washed twice with 200-ml portions of ethyl acetate. After cooling the aqueous phase in an ice bath, the solution was adjusted to pH 3 with 50% aqueous citric acid and saturated with NaCl. The solution was then extracted with three 250-ml portions of ethyl acetate. The combined ethyl acetate extracts were washed twice with 250 ml of a saturated solution of sodium chloride and dried over sodium sulfate. Evaporation *in vacuo* yielded an oil (64.3 g). This oil was diluted with 40 ml of ether and warmed slightly. The product crystallized on seeding to yield 45.6 g (52%); dec 110–112°; $[\alpha]^{25}D$ −35.8° (*c* 1, H_2O). Recrystallization, if necessary, can be accomplished from ethyl acetate–benzene.

Anal. Calcd for $C_{11}H_{20}N_2O_5S$: C, 45.19; H, 6.89; H, 9.58; S, 10.96. Found: C, 45.30; H, 6.86; N, 9.54; S, 11.00.

(*N*-Hydroxysuccinimido)-*N-tert*-butoxycarbonyl-*S*-acetamidomethyl-L-cysteinate. *N*-Boc-*S*-Acm-Cys (7.3 g, 0.025 mol) was dissolved in 100 ml of peroxide-free THF in a dry flask. The solution was cooled in an ice–salt bath and 2.9 g (0.025 mol) of *N*-hydroxysuccinimide was added. When solution was complete, 5.66 g (0.0275 mol) of dicyclohexylcarbodiimide was added and the mixture was stirred 1 hr at ice–salt bath temperature and then refrigerated overnight (5°). The mixture was filtered and the filtrate evaporated *in vacuo* to give a white solid which was dissolved in ethyl acetate, washed once with 1 *N* $NaHCO_3$, three times with a saturated solution of NaCl, dried over Na_2SO_4, and evaporated *in vacuo.* The resulting amorphous solid was dissolved in a minimum of $CHCl_3$ at room temperature and ether was added not quite to the cloud point. The product crystallized on standing: 8.1 g (80% yield); mp 106–108°; $[\alpha]^{25}D$ +43.0° (*c* 1, $CHCl_3$). The infrared spectrum showed carbonyl peaks at 5.52, 5.61, 5.74, 5.83, and 5.98 μ (Nujol). The nmr spectrum was consistent with the desired structure.

Anal. Calcd for $C_{15}H_{23}N_3O_7S$: C, 46.25; H, 5.95; N, 10.79; S, 8.23. Found: C, 46.34; H, 6.13; N, 10.97; S, 8.49.

***N*-Cbz-*S*-Acm-L-Cys** was prepared by the reaction of Acm-Cys with benzyl chloroformate in aqueous medium at pH 10 and isolated according to the usual procedures for such derivatives.[21] The product was isolated as an oil which was used directly to prepare the hydroxysuccinimide ester.

(*N*-Hydroxysuccinimido)-Cbz-*S*-Acm-L-cysteinate was prepared in the same manner as the Boc derivative. The product was crystallized once from ethyl acetate and recrystallized from ethanol to give mp 152–154°. The ir spectrum was consistent with this structure.

Anal. Calcd for $C_{18}H_{21}N_3O_7S$: C, 51.05; H, 5.00; N, 9.92; S, 7.57. Found: C, 50.86; H, 4.82; N, 9.66; S, 8.32.

Rate of racemization of (*N*-hydroxysuccinimido)-*N*-Cbz-*S*-Acm-Cys in the presence of triethylamine was determined following the procedure of Kovacs, *et al.*[9] Using an ester concentration of 0.028 *M* and triethylamine concentrations of 0.47 and 0.90 *M* gave first-order rate constants of 1.25×10^{-3} and 2.52×10^{-3} sec^{-1}, respectively, at 23°. Thus, the average second-order rate constant was $27 \pm 2 \times 10^{-4}$ M^{-1} sec^{-1}.

Gly-*S*-Acm-L-Cys. *S*-Acm-Cys (3.84 g, 0.02 mol) was dissolved in 200 ml of water at 0–3° and the pH of the solution was adjusted to 9.5 with saturated $Ba(OH)_2$. Thiazolidine-2,5-dione[22] (2.57 g, 0.022 mol) was added with stirring and the pH was maintained at 9.5 by the addition of saturated $Ba(OH)_2$. When no further addition of base was required, concentrated H_2SO_4 was added to bring the solution to pH 5.5. The precipitated barium sulfate was filtered and the solution evaporated *in vacuo.* The resulting solid, 4.7 g (94%), was shown by tlc (butanol–acetic acid–water (10:2:3)) to contain a small amount of impurity, probably Gly-Gly-(Acm)-Cys.

This impurity was removed by dry column chromatography[23] using silica gel H as absorbent and *n*-butyl alcohol–acetic acid–water (10:2:3) as eluent. The fractions containing pure product as determined by tlc were pooled and evaporated to dryness *in vacuo.* The resulting material was crystallized by dissolving in a minimum of hot water, adding 5–10 vol of methanol, warming the solution slightly, and adding ether to the cloud point. A sample dried at 100° gave dec 189°.

Anal. Calcd for $C_8H_{15}N_3O_4S$: C, 38.54; H, 6.06; N, 16.86; S, 12.83. Found: C, 38.33; H, 5.87; N, 16.79; S, 12.55.

Removal of the Acetamidomethyl Group. (a) *S*-Acm-Cys (96.1 mg, 0.5 mmol) was dissolved in 10.0 ml of water and the pH adjusted to 4.0. Mercuric acetate (159.3 mg, 0.5 mmol) was added with stirring and the solution readjusted to pH 4.0. The mixture, containing some precipitated material, was stirred 1 hr at room temperature. In a separate study, samples were removed at shorter times. Tlc of these samples indicated 90% reaction after 15 min. At pH 2, 50% reaction occurred in 15 min. The mixture was diluted with water, H_2S bubbled into the mixture to precipitate Hg(II), and the mixture filtered. Tlc (*n*-butyl alcohol–acetic acid–water (10:2:3)) showed the removal of the acetamidomethyl group to be complete. After air oxidation at pH 7 in the presence of a small piece of copper wire, the only detectable, ninhydrin-responding component was shown to be cystine by tlc. Tlc in the above system showed the oxidation to be complete. A quantitative ninhydrin assay[24] showed the yield to be greater than 98%.

(20) We are indebted to Dr. R. S. Dewey of these laboratories for the development of this procedure.

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(b) Gly-*S*-Acm-Cys. A solution of 310 mg (1.25 mmol) of Gly-*S*-Acm-Cys in 10 ml of water was adjusted to pH 4 with acetic acid. Mercuric acetate (398 mg, 1.25 mmol) was added and the resulting solution stirred 1 hr at room temperature. Hydrogen sulfide was bubbled into the solution to precipitate Hg(II) and the mixture was filtered. The filtrates were adjusted to pH 7 and air bubbled through the solution overnight in the presence of copper wire. The solution was then evaporated *in vacuo*. The resultant solid was dissolved in water and several volumes of ethanol were added to give crystalline diglycylcystine, 106 mg (48%). The ir and nmr spectra of this material were identical with those of an authentic sample.

Tlc showed the only product, detectable by ninhydrin, to be diglycylcystine. Nmr of a crude sample of this material showed peaks for acetamidomethanol in addition to those of diglycylcystine.

Stability of the Acetamidomethyl Blocking Group. Samples of *S*-Acm-Cys and Gly-*S*-Acm-Cys were treated under the conditions described in Table I. The reactions were analyzed by tlc developed with ninhydrin. The results are summarized in Table I. In those cases where partial reaction is indicated, the amount was not determined because these conditions are deemed unsatisfactory. Acm-Cys was stable in HBr–HOAc while Gly-*S*-Acm-Cys showed evidence of decomposition after 5 hr at 25°.

***tert*-Butyloxycarbonyl-L-threonyl-L-aspartyl-*S*-(Acm)-L-cysteinyl-L-arginine Ethyl Ester (Ribonuclease Fragment 82–85).** To a solution of 11.04 g (0.040 mol) of L-arginine ethyl ester dihydrochloride in 240 ml of 0.2 *N* $NaHCO_3$ at pH 8.0 was added a solution of 21 g (54 mmol) of (*N*-hydroxysuccinimido)-*N*-Boc-*S*-Acm-cysteinate in 100 ml of ethanol with stirring at 25°. The pH was maintained at 8.0 by the addition of a solution of 3 *N* NaOH. When base consumption ceased, the pH was adjusted to 4.0, and the reaction mixture was filtered and evaporated to dryness. The crude product was dissolved in $CHCl_3$ and filtered to remove salt. The filtrate was dried over Na_2SO_4 and evaporated *in vacuo* to give a viscous oil. This oil was chromatographed on a 1600-g silica gel column using butanol–acetic acid–water (10:2.3:6) as eluent, taking 100-ml fractions. Fractions 23–26 contained the pure product as shown by tlc in the same system (R_f 0.62). These were combined, evaporated to dryness and triturated with ether. (Fractions 27–29 contained another 5.7 g. These fractions were not used due to the presence of an unknown impurity.) The resulting white solid was suspended in 500 ml of ethyl acetate and anhydrous HCl was bubbled into the mixture for 10 min (to saturation) to remove the Boc group. During the addition of HCl, the suspended material dissolved and the dipeptide ester immediately began to precipitate as the hydrochloride salt. The solution was flushed for 15 min with a stream of nitrogen and filtered to give 11.87 g (66%) of *S*-Acm-cysteinylarginine ethyl ester dihydrochloride. The product (26.4 mmol) was dissolved in 264 ml of 1 *M* potassium borate buffer at 0° in a Waring blender and the pH was raised to 10.0. Aspartic acid *N*-carboxyanhydride[25] (4.831 g, 30.3 mmol) was added over a period of 1 min and the pH was maintained at 10.0 by the addition of 50% KOH from a pipet. The pH was adjusted to 3.0 with concentrated H_2SO_4; the solution was filtered and evaporated to dryness *in vacuo*. The resulting solid was extracted three times with 500-ml portions of ethanol and filtered to remove salt. The ethanol extracts were combined and evaporated to dryness *in vacuo* and flushed several times with ethanol to remove borate as ethyl borate leaving 12.0 g of crude aspartyl-*S*-Acm-cysteinylarginine ethyl ester (probably as the bisulfate salt). This tripeptide was dissolved in 227 ml of water and the pH was adjusted to 8.0 with sodium hydroxide. Boc-threonine-*N*-hydroxysuccinimide ester (8.61 g, 27.2 mmol) was added and the pH 8.0 maintained by the addition of 2.5 *N* NaOH. When base consumption ceased, the pH was adjusted to 8.5 and the solution was extracted five times with 300-ml portions of *n*-butyl alcohol saturated with water. The combined extracts were evaporated to dryness *in vacuo* to give 10.0 g of a mixture which was shown by tlc (*n*-butyl alcohol–acetic acid–water (10:2.3:6)) to contain three components—*tert*-Boc-L-threonyl-L-aspartyl-*S*-Acm-L-cysteinyl-L-arginine ethyl ester (R_f 0.52), an unknown peptide impurity (R_f 0.57), and *tert*-Boc-threonine (R_f 0.95). This mixture was dissolved in 50 ml of a mixture of chloroform–ethanol–water (50:50:10), adsorbed on an 800-g silica gel "dry column"[23] and eluted with the same solvent mixture (50-ml cuts were taken). Cuts 21–31 were found to contain the product and Boc-threonine but were free of the unknown peptide impurity. These cuts were combined and evaporated to dryness *in vacuo*. A 40-plate countercurrent extraction of this material in *n*-butyl alcohol–acetic acid–water (4:1:5) using 50 ml of each layer per tube gave complete separation of the peptide from Boc-threonine. The fractions containing only peptide as shown by tlc were combined, evaporated to dryness *in vacuo*, and triturated with ether to give 3.17 g (4.57 mmol; 11% yield based on arginine ethyl ester); $[\alpha]^{25}_{589}$ $-65.9°$ (1% *N* acetic acid). Amino acid analysis after acid hydrolysis gave $Thr_{1.02}Asp_{1.00}Arg_{0.99}$. A sample air-oxidized at pH 10 after addition of 1 *N* NaOH to the acid hydrolysate gave $Thr_{1.01}Asp_{1.01}Arg_{0.98}0.5Cys_{0.93}$.

Anal. Calcd for $C_{27}H_{48}N_8O_{11}S$: C, 46.81; H, 6.98; N, 16.17; S, 4.63; Found: C, 46.55; H, 7.00; N, 16.16; S, 4.50.

Octa-*S*-Acm-octahydro-*S*-protein. Preparation. *S*-Protein (200 mg) was dissolved in 6 ml of 8 *M* urea containing 0.7 ml of β-mercaptoethanol in a nitrogen atmosphere. The pH was adjusted to 8.65 with 40% aqueous methylamine. After standing 1 hr at room temperature, the solution was cooled to $-15°$. The reduced protein was precipitated by the addition of 20 ml of ethanol–1 *N* HCl (40:1). The precipitate was washed twice with this solvent and three times with absolute ethanol to give 147 mg (12.7 μmol). In subsequent preparations higher yields of monomeric product were obtained if these washing solvents contained 0.5% acetic acid to depress the oxidation of the thiol. Hydroxymethylacetamide (11 mg, 124 μmol) was added and the mixture was dissolved in 1 ml of anhydrous HF at $-78°$. The solution was warmed to 0° in an ice bath and allowed to stand for 0.5 hr. The HF was removed by evaporation under a stream of N_2 and finally *in vacuo*. The protein was washed three times with 2-ml portions of ethanol and dissolved in 20 ml of 50% aqueous acetic acid. This material was purified by chromatography on a 5 × 100 cm Sephadex G-75 column using 50% acetic acid as eluent taking 15-ml fractions. Fractions 48–59 (the same elution volume as *S*-protein on this column) were pooled and lyophilized (68 mg). A material of higher molecular weight (dimer) was obtained on lyophilization of fractions 37–44 (62 mg). This material showed no thiol when tested according to the method of Ellman.[26]

Table II. Amino Acid Analysis of Acetamidomethylated *S*-Protein

	Monomer				
	Acid	Enzyme		Dimer	
Amino acid	(20 hr)	*a*	*b*	enzyme[a]	Theory
Lys	7.7	8.3	7.7	*c*	8
His	2.7	2.8	2.9	*c*	3
Arg	2.8	3.0	3.1	*c*	3
Acm-Cys			7.5		8
Acm-Cys + Asp		12.3	11.5	10.0	12
Asp			4.0		4
Asn		*c*	8.4	*c*	10
Asp + Asn	14.2				14
Glu		3.1	3.5	3.1	3
Gln		*c*	5.5	*c*	6
Glu + Gln	9.1				9
Ser	12.7	*c*	12.2	*c*	12
Thr	8.0	*c*	*c*	*c*	8
Pro	7.5[d]	2.04	4.2	4.2	4
Gly	3.3	2.7	2.6	3.1	3
Ala	7.2	7.3	6.9	6.8	7
0.5Cys	1.2	0	0	0.6	0
Val	9.0	8.9	9.0	9.1	9
Met	2.8	3.0	2.8	2.5	3
Ileu	2.4[e]	2.8	2.9	2.9	3
Leu	2.1	2.3	2.0	2.2	2
Tyr	5.4	5.0	5.9	5.6	6
Phe	2.0	2.0	2.1	2.0	2

[a] Papain followed by aminopeptidase M. Sodium[6] column only. [b] Pronase followed by aminopeptidase M. Both sodium[6] and lithium[9] columns. [c] Not determined. [d] Includes some cysteine. [e] Expected to be low after 20 hr of hydrolysis.

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A sample of the dimer (285 mg) was reduced and alkylated as described above. After purification on Sephadex G-75, additional monomer (95 mg) was obtained. Dimer (103 mg) was also recovered in this experiment. When this reaction was run using [^{14}C]acetamidomethanol (13.7 μCi/mmol) prepared from ^{14}C-labeled acetamide, the monomer was found to have a specific activity of 110 μCi/mmol corresponding to the incorporation of eight residues of Acm. The dimer had a specific activity of 167 μCi/mmol or 14 residues of Acm.

Enzymatic Degradation. (a) A sample of octa-*S*-Acm-octahydro-*S*-protein was hydrolyzed with papain followed by aminopeptidase-M according to the method of Brewer, *et al.*[27] The results are given under column a in Table II. The sodium citrate column[6] was used only for the analysis. (b) A sample was also hydrolyzed using pronase followed by aminopeptidase-M according to the method described by Bennett, *et al.*[28] The results are given under column b in Table II.

Reconversion to Ribonuclease-*S*′. Octa-*S*-Acm-octahydro-*S*-protein (12 mg) was dissolved in 0.75 ml of 50% aqueous acetic acid and a solution of mercuric acetate (15.1 mg) in 0.17 ml of 50% acetic acid was added. After standing for 70 min at room temperature, β-mercaptoethanol (0.9 ml) was added. The mixture was stirred in a N_2 atmosphere for 19 hr. The mercuric ion and excess β-mercaptoethanol were then removed by chromatography on a 3 × 29 cm column of Sephadex G-25 (fine) using 0.1 *N* acetic acid as eluent. The fractions containing protein were combined and diluted to 25 ml with 0.1 *N* acetic acid. The solution was shown to contain 9 mg of *S*-protein by the method of Lowry[29] using a solution of *S*-protein as standard. A solution of *S*-peptide (5 mg), $NaH_2PO_4 \cdot H_2O$ (1.24 g) and Na_2HPO_4 (0.852 g) in 25 ml followed by β-mercaptoethanol (10 μl) was added and the pH adjusted to 6.5 with 40% aqueous methylamine. The total volume was brought to 75 ml with H_2O, and four drops of $CHCl_3$ were added to prevent bacterial contamination. After standing 14 days in the presence of air, the yield of ribonuclease-*S* activity was determined essentially according to the method of Anfinsen, *et al.*[30] The yield of enzymatic activity was equal to 3.2 mg of ribonuclease-*S* (30%). A precipitate (4.6 mg) was also present. Copper sulfate (75 μg) was added to increase the rate of oxidation of the remaining thiol and the solution was allowed to stand to complete disappearance of thiol.[26] No change in enzymatic activity occurred during this time. EDTA solution (1 ml of 0.1 *M*) was added. The solution was lyophilized and the salts removed by passage through a 2.5 × 28 cm Bio-Gel P-6 (200–400 mesh) column using 1 *M* NH_4OAc (pH 7.5) as eluent. The protein-containing fractions were combined and lyophilized (3.7 mg having enzymatic activity equal to 1.6 mg of ribonuclease-*S*). This material was purified by chromatography on a 1.5 × 43 cm carboxymethyl Sephadex C-50 column using 0.2 *M* sodium phosphate buffer of pH 6.47 as eluent. The product was eluted at 168 ml, the same elution volume as ribonuclease-*S*. This material (1.3 mg) had a specific activity of 90%. A by-product of lower specific activity was eluted first and overlapped slightly with the product (0.6 mg, 45% active).

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A Chlorine-35 Nuclear Magnetic Resonance Study of Dodecyl Sulfate Binding to Bovine Serum Albumin[1]

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Abstract: The nuclear magnetic resonance spectrum of chlorine-35 ions in the presence of bovine serum albumin is broadened considerably. The chloride interaction is presumably at centers of positive charge with no metal ion being necessary. Binding of dodecyl sulfate and other anions to albumin decreases this interaction as shown by a decrease in the chlorine resonance line width. By monitoring the chlorine-35 line width when adding detergent, a titration curve is obtained which shows that most of the chloride binding is eliminated in the first 5–10 equiv of added detergent. This decrease occurs when the dodecyl sulfate is binding at the eight–ten strong binding sites. Presumably a center of positive charge on the protein where chloride binds is being neutralized. Higher concentrations of dodecyl sulfate are needed to remove all chloride interaction. Titration of albumin labeled at the free cysteine with a mercuric ion is different from the titration of free albumin, demonstrating that the titration curve is sensitive to changes in the protein. Acetimidation of the lysines in bovine serum albumin, which does not change the net charge, does little to change the detergent binding to the protein. Anions other than dodecyl sulfate also give titration curves. Hexyl, octyl, and decyl sulfate are not as effective in reducing chloride binding at similar concentrations.

Nuclear magnetic resonance spectroscopy of quadrupolar ions is rapidly becoming a valuable tool in the study of biological systems. Chlorine-35,[2,3] calcium-43,[4] sodium-23,[5] and magnesium-25[6] are some

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