2-Sulphobenzyl, a New Solubilizing and Reversible Protecting Group for Cysteine in Proteins

ITS SCOPE AND LIMITATIONS

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S-2-Sulphobenzylcysteine and *S*-2-(sulphomethyl)benzylcysteine are prepared by alkylation of cysteine with ω -toluenesultone and 2,3-benzo-1,4-butanedisultone respectively. Owing to the presence of the sulphonic acid group, these protected cysteine derivatives are extremely water-soluble and are stable to acid hydrolysis. The groups can be removed by treatment with sodium in liquid NH_3 . Reduction with tributylphosphine and simultaneous alkylation of insulin with toluenesultone under mild conditions (pH 8.3, aq. 50% propanol) gives the fully *S*-substituted derivatives in excellent yield; they can be separated by isoelectric precipitation of the *S*-sulphobenzylated B-chain. Treatment of the latter with sodium in liquid NH_3 led simultaneously to the removal of the protecting groups and to the well-documented cleavage at the threonine–proline bond which can be prevented by addition of sodium amide. When deprotected A-chain was recombined with B-chain, insulin was isolated in the same yield and with the same degree of biological activity as that in the control experiment.

In introducing *S*-3-sulphopropyl (Rüegg & Rudinger, 1974*b*, 1977*b*) as a modifying group for the cysteine residues of proteins we demonstrated the advantages of sulphoalkylation for such derivative formation. That study showed that the reaction of a γ -sultone with thiol groups proceeds readily under mild conditions and with a high degree of selectivity. Furthermore, the sulphonyl grouping of the reagent serves the dual function of activating the terminal methylene group and, after reaction, providing the very polar sulphonic acid grouping as side-chain substituent.

In the preceding paper (Rüegg *et al.*, 1979) we set out the features generally required of a protecting group and pointed out that the conferment of good solubility properties on the modified polypeptide chain is integral to them. In the polar, possibly aqueous, solvents needed for the manipulation of proteins or fragments derived from them, polar pro-

tecting groups would seem to be very desirable, if not essential. In this connection it should be pointed out that sulpho, a group with very good solubilizing properties, has been successfully used for *S*-protection in some partial syntheses (Weinert *et al.*, 1969, 1971; Rees & Offord, 1976*a,b*).

Our earlier study showed that the *S*-3-sulphopropyl derivatives of insulin chains have similarly good solubility properties in aqueous media. It therefore seemed highly desirable to combine the undoubted advantages of *S*-sulphoalkylation with structural requirements which would permit cleav-

Abbreviations used: A, A-chain of insulin without S atoms at positions 6, 7, 11 and 20; B, B-chain without S atoms at positions 7 and 19; Sb, 2-sulphobenzyl; Aet, 2-aminoethyl.

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age of the modifying group. This would lead to the design of reversible protecting groups for thiols which would be suitable for the partial synthesis of proteins. In an attempt to do this we examine, in this paper, the usefulness of ω -toluenesultone (I), and also that of 2,3-benzo-1,4-butanedisultone, for the reversible protection of cysteine in proteins in the form of its 2-sulphobenzyl (II) and 2-(sulphomethyl)-benzyl derivatives.

Experimental

Materials

Sources of common chemicals, tributylphosphine, gel-filtration media and ion-exchange resins as well as the procedure for the purification of insulin are given in the preceding paper (Rüegg *et al.*, 1979). ω -Toluenedisultone (3*H*-2,1-benzoxathiole 1,1-dioxide) was prepared from benzaldehyde-2-sulphonic acid (Helberger *et al.*, 1949). The product was recrystallized from toluene to give material with m.p. 113–114°C, which was homogeneous on t.l.c. in the solvent methanol/ethyl acetate (1:3, v/v). 2,3-Benzo-1,4-butanedisultone was prepared from σ -xylene dibromide by a procedure appropriately modified from that of Helberger & Sproviero (1963). The product was recrystallized from toluene/light petroleum (b.p. 40–65°C) to give analytically pure material, with m.p. 113–114°C, which was homogeneous on t.l.c. in the solvent methanol/chloroform (1:9, v/v).

Note of caution. More well-known sultones, such as 1,4-propanedisultone, are known to be carcinogenic (McCann *et al.*, 1975); we advise that the above sultones are handled with great care.

Methods

Optical rotations were measured with a Perkin-Elmer model 141 photoelectric polarimeter. All evaporations were carried out under reduced pressure in a rotary evaporator at bath temperatures not exceeding 35°C.

Thin-layer electrophoresis. This was carried out in a moist-chamber apparatus (Camag A.G., Muttenz, Switzerland). A potential gradient of about 25 V/cm was applied for 2 h to the modified insulin chains (samples 10–50 nmol in 5–10 μ l). The support was cellulose acetate on polyester foil (TLC-Ready Plastic Sheets F-1440/45ac; Schleicher und Schüll, Dassel, Germany), the electrolyte was 2.4 M-formic acid in 7 M-urea (pH 2.2) (cf., e.g., Krail *et al.*, 1971); after being dried at 120°C for 10 min the plates were washed with ethanol to remove urea and sprayed with Pauly reagent.

Desalting by gel filtration. Salts were removed by chromatography on Sephadex G-25 (Rüegg *et al.*, 1979).

Amino acid analyses. Conditions for hydrolysis and analysis are given in the preceding paper (Rüegg *et al.*, 1979). *S*-2-Sulphobenzylcysteine is eluted from the long column of the amino acid analyser (Spackman, 1967) slightly behind cysteic acid and the colour constant was determined to be 1.01 times that for aspartic acid.

Reductions with sodium in liquid NH₃. These were carried out by the extractive procedure of Nesvadba & Roth (1967) in the type of apparatus described by them. Persistence of the blue colour for 2–5 s was taken to indicate complete reduction: the colour was then discharged by the immediate addition of NH₄Cl. The solution was frozen and freeze-dried and the residue was dried over H₂SO₄.

Experiments with liquid HF were carried out in an apparatus by the procedure described by Sakakibara *et al.* (1967). The apparatus was obtained from Toho-Kasei Co., Kami Fukuido 2, Hagashisumiyoshi, Osaka, Japan.

S-2-Sulphobenzyl-L-cysteine. L-Cysteine hydrochloride (788 mg; 5 mmol) in 0.5 M-NaHCO₃/propanol (1:1, v/v; 50 ml) was treated, under N₂, with ω -toluenedisultone (936 mg; 5.5 mmol). The mixture was stirred for 14 h, during which time most of the rather sparingly soluble sultone dissolved. After filtration, the solution was adjusted to pH 4 with acetic acid and applied to a column (2.6 cm $\times$ 12 cm) of Dowex 50W (X8; H⁺ form; 100–200 mesh). The column was washed with water until the *A*₂₈₀ reached baseline and the combined washings were evaporated under reduced pressure. The residue was crystallized from 25 ml of boiling water to give 1.2 g (81%); $[\alpha]_D^{25}$ 2.9° (c 2.2 in 1 M-NaOH) (Found: C, 41.1; H, 4.5; N, 4.7; S, 21.9; C₁₀H₁₃NO₃S₂ requires C, 41.2; H, 4.5; N, 4.8; S, 22.0%).

S-2-(Sulphomethyl)benzylcysteine. This was prepared and purified in the same way as *S*-2-sulphobenzylcysteine, but the time of reaction was extended to 2 days. Material which on electrophoresis was homogeneous and behaved identically with *S*-2-sulphobenzylcysteine was used in the experiment with HF.

Reduction and *S*-2-sulphobenzylation of insulin

Insulin (574 mg; 100 μ mol) in 0.5 M-NaHCO₃/propanol (1:1, v/v; 100 ml) was treated, under N₂, with tributylphosphine (330 μ mol) and ω -toluenedisultone (204 mg; 1200 μ mol). The mixture was stirred for 14 h, during which time practically all of the sultone went into solution. After filtration, the solution was concentrated to about 40 ml and transferred to the 100 ml beaker of a Hollow Fiber Dialyzer b/HFD-1 (Dow) where it was dialysed against demineralized water (9 litres) during 2.5 h. The dialysis residue was freeze-dried to give 661 mg (97%) of a 1:1

mixture of A(SSb)₄ and B(SSb)₂. Amino acid analysis is given in Table 1 (column 2).

Separation of S-2-sulphobenzylated A- and B-chains

A portion (500mg) of the above mixture of chain derivatives was suspended in water (150ml). A 1M solution of NH₃ was added slowly and with stirring until the protein dissolved; the pH was 8.5. The pH of the solution was then gradually lowered to 6.5 by dropwise addition of 1M-acetic acid and the precipitate was removed by centrifugation at 7000rev./min for 10min. The supernatant was concentrated to 10ml by flash evaporation, adjusted to pH8.5 with 1M-NH₃ and applied to a column (2.5cm×82cm) of Sephadex G-50 fine, which had been equilibrated with 0.1M-NH₄HCO₃. Elution at 45ml/h with the same buffer gave a very small peak at 95ml (<1% of total), which was discarded, and the main peak at 130ml. After pooling of the fractions and freeze-drying 171mg (77%) of A(SSb)₄ was recovered. For amino acid analysis, see Table 1 (column 3).

The precipitate obtained by centrifugation was suspended in water (80ml) and brought into solution by the addition of 1M-NH₃ to give pH8.5. Lowering of the pH to 5–6 by the gradual addition of 1M-acetic acid again led to formation of a precipitate which was collected by low-speed centrifugation as before. Dissolution and precipitation were repeated twice before the precipitate was dissolved in water (30ml) by adjusting the pH to 8.5 with 1M-NH₃, and freeze-dried to give 238mg (86%) of B(SSb)₂. The amino acid analysis is shown in Table 1 (column 4).

Reduction of S-2-sulphobenzylcysteine and oxidation to cystine

S-2-Sulphobenzylcysteine (582mg; 2mmol) was reduced in liquid NH₃ (150ml), 183mg (8mg-atoms) of sodium being consumed. The blue colour, indicative of complete reduction, was quenched with NH₄Cl (50mg) and the solution was frozen and freeze-dried. The residue was dissolved in water (7ml) and oxidized by the addition of 1,2-di-iodo-methane (30.1mg; 1.05mmol) in ethanol (3ml) by the procedure of Weygand & Zumach (1962). The cystine formed was filtered off, washed with propan-2-ol and dried to yield 190mg (79%).

$[\alpha]_D^{25} - 227^\circ$ (c 1.9 in 1M-HCl). This value compares well with literature values, e.g. $[\alpha]_D^{25} - 213.7^\circ$ (c 1.9 in 1M-HCl) (Weygand & Zumach, 1962).

Reduction of A(SSb)₄ and its conversion into A(SAet)₄

A(SSb)₄ (15.5mg; 5.5μmol) was reduced with sodium in liquid NH₃ (30ml) as described above. Ammonia was removed by freeze-drying and the residue was dissolved in 8M-urea (10ml) to give a solution of pH8.4. Tributylphosphine (about

150μmol) was added as oxygen scavenger together with ethyleneimine (100μl; 950μmol) (Cole, 1967). After 1h, the solution was desalted on a column (1.7cm×28cm) of Sephadex G-25 (medium) in 0.01M-NH₄HCO₃. Freeze-drying of the protein peak gave 10.5mg (76%) of A(SAet)₄. Amino acid analysis is shown in Table 1 (column 5).

Reduction of A(SSb)₄ and its conversion into A(SSO₃H)₄

A(SSb)₄ (76mg; 27μmol) was reduced with sodium in liquid NH₃ (100ml) and the NH₃ was removed by freeze-drying. The residue was dissolved in 0.1M-Tris/HCl/8M-guanidine hydrochloride, pH8.5 (20ml). To this solution, sodium sulphite (1g) and sodium tetrathionate (0.5g) were added and the pH was re-adjusted to 8.5. After being left at room temperature for 24h, the solution was diluted with acetic acid (5ml) and desalted by gel filtration in 50% acetic acid. Flash evaporation and freeze-drying of the protein eluate gave 67.5mg of A(SSO₃H)₄. The amino acid analysis is presented in Table 1 (column 7).

Reduction of B(SSb)₂ and its conversion into B(SSO₃H)₂

B(SSb)₂ (76mg; 20μmol) was added to either liquid NH₃ (150ml) or liquid NH₃ (150ml) containing a suspension of sodium amide (100mg). Reduction with sodium was carried out as for A(SSb)₄ although the end point was less easy to determine in the presence of sodium amide (slow appearance of a greenish colour). After the colour had been discharged by the addition of a few crystals of NH₄Cl, the solution was freeze-dried. The residue was dissolved in 0.1M-Tris/HCl/8M-guanidine hydrochloride, pH8.5, and converted into B(SSO₃H)₂ in a manner quite analogous to that described above for the preparation of A(SSO₃H)₄. After being desalted in 50% acetic acid, the product was isolated by freeze-drying; the yield was about the same in both experiments (58mg; 81%).

Each of these materials was purified on a column (2.6cm×56cm) of CM-cellulose. Elution at 33ml/h was carried out with 0.04M-sodium acetate/8M-urea (adjusted to pH4.0 with acetic acid) (Katsoyannis *et al.*, 1967). The fractions were pooled as indicated in Fig. 1, desalted by gel filtration in 50% acetic acid and isolated by evaporation and freeze-drying. From the preparation in which reduction had been carried out in the presence of sodium amide, the yield of B(SSO₃H)₂ was 28.7mg (41%).

Chain recombination and crystallization of insulin

A(SSO₃H)₄ (66.5mg; 25μmol), obtained after de-protection, was recombined with B(SSO₃H)₂ (89mg;

Table 1. *Amino acid analysis*
The value for proline includes values of cysteine.

Material	...	Insulin reference	Composition (residues/peptide)									
			A(SSb) ₄ B(SSb) ₂	A(SSb) ₄	B(SSb) ₂	A(SAet) ₄ from A(SSb) ₅	A(SSO ₃ H) ₄ 'native'	A(SSO ₃ H) ₄ from A(SSb) ₇	B(SSO ₃ H) ₂ 'native'	B(SSb) ₂ 9	Insulin from A(SSb) ₁₀	
Number	...	1	2	3	4	5	6	7	8	9	10	
Lys		1.01	1.01	0.01	0.98	0	0	0	1.00	0.98	0.98	
Cys(Aet)		—	—	—	—	3.94	—	—	—	—	—	
His		1.91	1.96	0.01	1.87	0	0	0	1.93	2.03	1.89	
NH ₃		5.99	5.68	2.04	9.06	5.40	3.49	3.64	2.50	7.50	8.62	
Arg		0.97	0.99	0.01	1.00	0	0	0	0.97	1.00	1.03	
Cys(Sb)		—	6.09	3.94	2.01	0.07	—	0.03	—	0.23	—	
Asp		2.97	3.01	1.99	1.08	2.10	2.04	1.96	1.04	0.97	3.08	
Thr		0.95	0.97	0.01	0.95	0	0	0	0.97	0.91	0.94	
Ser		2.69	2.77	1.77	0.98	1.64	1.67	1.73	0.94	0.86	2.73	
Glu		7.04	7.06	3.98	3.30	3.97	4.09	4.03	3.10	3.20	7.06	
Pro		1.03	0.97	0	0.99	0	0.80	1.07	1.48	1.10	0.93	
Gly		4.04	3.97	0.97	2.97	0.98	0.98	1.00	3.04	2.97	3.96	
Ala		2.96	3.03	1.03	2.03	1.02	1.02	1.00	1.96	2.03	3.04	
‡(CyS) ₂		5.54	0	0	0	0	0.44	0.62	0.22	0.68	4.22	
Val		4.54	4.74	1.64	2.99	1.87	1.66	1.68	2.68	2.56	4.73	
Ile		0.68	0.65	0.62	0.02	0.82	0.64	0.63	0	0	0.71	
Leu		6.05	6.05	2.04	4.10	2.10	1.97	2.06	4.04	4.21	6.12	
Tyr		3.80	3.97	1.92	1.99	1.96	1.80	1.88	1.92	1.86	3.90	
Phe		2.92	2.97	0.03	2.92	0	0	0	2.84	2.99	2.93	

25 μ mol), which had been prepared by direct sulphitolyis of insulin (Katsoyannis *et al.*, 1967). A control experiment in which the *S*-sulpho derivatives of both chains had been obtained by direct sulphitolyis of insulin was run in parallel. The insulin formed was purified by gel filtration and chromatography on CM-cellulose (Fig. 2) as previously described (Rüegg & Gattner, 1975). Insulin was crystallized from citrate buffer containing 16% acetone as described (Kung *et al.*, 1966).

Bioassay

The glycolytic activity of the insulin preparations was measured by means of the isolated rat epididymal fat-pad assay described by Froesch *et al.* (1963).

Results

Introduction of the S-2-sulphobenzyl and the S-2-(sulphomethyl)benzyl protecting groups

S-2-Sulphobenzylcysteine was obtained in excellent yield from the reaction of cysteine with ω -toluenesultone in aqueous, weakly alkaline solution at room temperature. This derivative is very water-soluble, is not retained by a sulphonic acid ion-exchange resin and moves anodically in electrophoresis even at pH1.6. It is completely stable to the conditions of acid hydrolysis and is eluted at a position very close to that of cysteic acid from the long column of the amino acid analyser. As expected, 2,3-benzo-1,4-butanedisultone reacted more slowly with the thiol group of cysteine and *S*-2-(sulphomethyl)benzylcysteine was found to possess physicochemical properties similar to those of *S*-2-sulphobenzylcysteine.

In the case of insulin, the disulphide bonds were first reduced with tributylphosphine as previously described (Rüegg & Rudinger, 1974a, 1977a). ω -Toluenedisultone was found to react specifically and in high yield with the thiol groups so generated. Since the phosphine does not react with the disultone under the conditions used, both reagents were added together, a 2-fold excess of the disultone being sufficient to give the *S*-2-sulphobenzylated A- and B-chains in excellent yield. Amino acid analysis of the 1:1 chain mixture which was obtained after dialysis and freeze-drying showed that reduction and *S*-alkylation were complete (Table 1, column 2). Furthermore, electrophoretic analysis of the mixture revealed two bands which migrated with mobilities about 90% those of the corresponding *S*-sulpho chains.

The solubilizing effect of the sulpho groups was remarkable. The A-chain derivative, to which four 2-sulphobenzyl groups are attached, was soluble in aqueous solution at concentrations up to 20 mg/ml

between pH2 and 11. The B-chain derivative, substituted with only two sulphobenzyl groups, precipitated from aqueous solutions below pH7.5. This insolubility in acid solution, which is typical of B-chain derivatives bearing a negatively charged *S*-substituent, provided a convenient means by which the A- and B-chain derivatives could easily be separated. The products obtained by threefold reprecipitation of B(SSb)₂ at pH5–6 were found to be cross-contaminated to the extent of only 1 and 3%, respectively, as judged by amino acid analysis (lysine and isoleucine as diagnostic amino acids) (Table 1, columns 3 and 4). A similar, though somewhat less clear-cut, separation has been carried out with the *S*-sulpho derivatives (Katsoyannis *et al.*, 1967).

Removal of the S-2-sulphobenzyl and the S-2-(sulphomethyl)benzyl protecting groups

Liquid HF, the use of which has been described for the removal of the *S*-benzyl group (Sakakibara *et al.*, 1967), did not cleave the *S*-2-sulphobenzyl group at all, even after a reaction time of 2 h at 20°C. Under the same reaction conditions and even after longer reaction times only about 10% cleavage of the *S*-2-(sulphomethyl)benzyl group was observed.

Both protecting groups can, however, be removed by sodium in liquid NH₃ (Sifferd & du Vigneaud, 1935). In order to avoid exposure of the peptide to an excess of sodium, the extraction technique of Nesvadba & Roth (1967) was used. Reduction of *S*-2-sulphobenzylcysteine in this way, followed by oxidation of the product, gave cystine in good yield and a state of high optical purity.

A quantitative measure of the completeness of reduction was provided by an experiment with the *S*-sulphobenzylated A-chain in which the product was aminoethylated (Cole, 1967). The amino acid analysis (Table 1, column 5) indicated less than 2% contamination with the protected derivative. Furthermore, in this and preparative experiments the value for alanine, the product of a possible desulphurization reaction (Katsoyannis, 1964; Shimonishi *et al.*, 1969), was not increased.

In preparative experiments the chains were converted, after reduction, into the *S*-sulpho derivatives. When the *S*-sulpho derivative of the A-chain was analysed, it showed the typical three bands on electrophoresis (Rüegg & Gattner, 1975) and had an amino acid composition in very good agreement with that of reference material (Table 1, columns 6 and 7). These results suggested that no further purification of this derivative was warranted before insulin resynthesis.

When the *S*-sulpho derivative obtained by reduction and oxidative sulphitolyis of B(SSb)₂ was chromatographed on CM-cellulose as described by Katsoyannis *et al.* (1967, 1971a), a large additional peak was eluted immediately before B(SSO₃H)₂

(Fig. 1b). Amino acid analysis of this material, which represented about 70% of the product, indicated the loss of threonine, proline, lysine and alanine from the B-chain *S*-sulpho derivative and suggested that the majority of the material had undergone reductive cleavage at the secondary amide bond between threonine-27 and proline-28 of the B-chain. The extent of cleavage was about the same as that found previously (Benisek *et al.*, 1967; Katsoyannis *et al.*, 1971a), even though the earlier workers exposed the peptide to sodium for a longer time.

Reduction of $B(SSb)_2$ with sodium in liquid NH_3 in the presence of sodium amide, an agent known to suppress cleavage at proline residues (Katsoyannis *et al.*, 1971b), gave a product which, after oxidative sulfitolysis, behaved chromatographically exactly like $B(SSO_3H)_2$ (Fig. 1c). After being desalted and freeze-dried it was found to co-migrate with reference material in electrophoresis and to show a full complement of the expected amino acids (Table 1, column 9). However, a small amount (10%) of *S*-2-sulphobenzylcysteine remained under these conditions, probably owing to the fact that the end point is rather indeterminate in the presence of sodium amide.

Fig. 1. Purification of *S*-sulpho derivatives of the insulin B-chain

S-Sulpho B-chain preparations which had been differently treated before sulfitolysis were chromatographed on CM-cellulose (column 2.6 cm $\times$ 56 cm) in 0.04 M-sodium acetate/8 M-urea, pH 4.0. (a) Material obtained by direct sulfitolysis of insulin; (b) material obtained by cleavage of $B(SSb)_2$ with sodium in liquid NH_3 in the absence of sodium amide; (c) material obtained after cleavage of $B(SSb)_2$ with sodium in liquid NH_3 in the presence of sodium amide. Horizontal lines indicate pooling of fractions.

Fig. 2. Final step in the purification of resynthesized insulin: a comparison of two preparations

Chromatography of material prefractionated by gel filtration was carried out on CM-cellulose in 0.1 M-acetic acid/7 M-urea, pH 3.9 (---, gradient 0–0.1 M-NaCl). Pure insulin is eluted as peak C and deamidated form(s) immediately precede it. (a) Both chains originate directly from insulin; (b) deprotected A-chain combined with B-chain obtained directly from insulin.

Resynthesis of insulin

No resynthesis experiments were performed with the B-chain that had been subjected to reduction with sodium in liquid NH_3 owing to the low recovery of the whole chain. The *S*-sulpho A-chain obtained after metal reduction and oxidative sulfitolysis was combined, in a 1:1 molar ratio, with the *S*-sulpho derivative of the B-chain which had been prepared directly from insulin. A control experiment in which both *S*-sulpho chains had been prepared directly from insulin was run in tandem. Chain recombination and successive fractionation of the products on Sephadex and CM-cellulose were carried out as described by Rüegg & Gattner (1975) (Fig. 2). After desalting, 9.9 mg (6.9% overall yield) of insulin was obtained from the preparation containing the desulphobenzylated chain and 12.5 mg (8.7%) in the control experiment. The results of amino acid analyses of the two insulin preparations were in good agreement (Table 1, columns 1 and 10) and electrophoresis gave identical results for both materials. The biological activity of insulin derived from the deprotected chain was 12 ± 5 units/mg, compared with 19 ± 6 units/mg for the control. Both materials could

be crystallized under the standard conditions. These results suggest that the deprotection of the A(SSb)₄ proceeds in a satisfactory manner.

Discussion

Our results indicate that in two important respects the 2-sulphobenzyl protecting group fulfils the promise of our earlier study (Rüegg & Rudinger, 1974b). As expected, *S*-sulphoalkylation takes place with high selectivity in aqueous, weakly alkaline solution. Since the sultone, like certain other alkylating agents (Rüegg & Rudinger, 1974a), is unaffected by tributylphosphine, reduction and alkylation were carried out in a single operation. It was found that a 10% excess of the phosphine and a 2-fold excess of the sultone were sufficient to give the *S*-protected insulin chains in almost quantitative yield.

Furthermore, the protecting group confers good solubility on the chain derivatives. In this regard it is much superior to the benzyl and other benzyl-based groups, the form of *S*-protection most commonly used for the insulin chains (see, e.g., Tsou *et al.*, 1961). Sulphobenzyl endows the A-chain derivative with better solubility properties, over a wider pH range than pyridylmethyl, the usefulness of which we discussed in the preceding paper (Rüegg *et al.*, 1979). Neither sulphobenzyl nor sulphomethylbenzyl, in which a methylene unit is formally inserted between the sulphonic acid grouping and the aromatic ring, can be cleaved by acidolysis or anhydrous HF. The sole means of removal is reduction with sodium in liquid NH₃. We have adopted the extraction procedure of Nesvadba & Roth (1967), which offers the advantage that the presence of an excess of sodium can be limited to a very short time or avoided altogether. Indeed it works well for the protected A-chain. The protecting groups were removed and the reduced chain was oxidized to give the *S*-sulpho derivative, which was analytically pure. Its recombination with a B-chain preparation gave insulin in a yield and state of purity which was comparable with that obtained with an A-chain that had not been subjected to protection and deprotection.

When the protected B-chain was treated with sodium in liquid NH₃, chain cleavage at the threonine-proline bond took place simultaneously with the removal of the protecting groups. Cleavage of acyl proline bonds in this way is, of course, well documented in the literature (see, e.g., Benisek *et al.*, 1967) and the reaction has, in fact, been exploited in synthesis. Katsoyannis *et al.* (1971a) have shown that the truncated B-chain can be recombined with A-chain to give an analogue of insulin which possessed biological activity similar to that of insulin itself. The same workers also demonstrated that this type of

cleavage can be suppressed if the reaction is carried out in the presence of sodium amide (Katsoyannis *et al.*, 1971b). We were able to confirm this finding in the case of the *S*-sulphobenzyl protected B-chain. Thus the major defect of the reductive cleavage can be overcome and the protecting group may be expected to be useful in synthesis, particularly in those instances where use needs to be made of its excellent solubilizing properties.

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References

- Benisek, W. F., Raftery, M. A. & Cole, R. D. (1967) *Biochemistry* **6**, 3780–3790
- Cole, R. D. (1967) *Methods Enzymol.* **11**, 206–208
- Froesch, E. R., Buergi, H., Ramseier, E. B., Bally, P. & Labhart, A. (1963) *J. Clin. Invest.* **42**, 1816–1834
- Helberger, J. H. & Sproviero, J. F. (1963) *Liebigs Ann. Chem.* **666**, 75–77
- Helberger, J. H., Manecke, G. & Heyden, R. (1949) *Liebigs Ann. Chem.* **565**, 22–35
- Katsoyannis, P. G. (1964) *Diabetes* **13**, 339–348
- Katsoyannis, P. G., Tometsko, A., Zalut, C., Johnson, S. & Trakatellis, A. C. (1967) *Biochemistry* **6**, 2635–2642
- Katsoyannis, P. G., Zalut, C., Harris, A. & Meyer, R. J. (1971a) *Biochemistry* **10**, 3884–3889
- Katsoyannis, P. G., Zalut, C., Tometsko, A., Tilak, M., Johnson, S. & Trakatellis, A. C. (1971b) *J. Am. Chem. Soc.* **93**, 5871–5877
- Krail, G., Brandenburg, D., Zahn, H. & Geiger, R. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 1595–1598
- Kung, Y.-T., Du, Y.-C., Huang, W.-T., Chen, C.-C., Ke, L.-T., Hu, S.-C., Jiang, R.-Q., Chu, S.-Q., Niu, C.-I., Hsu, J.-Z., Chang, W.-C., Chen, L.-L., Li, H.-S., Wang, Y., Loh, T.-P., Chi, A.-H., Li, C.-H., Shi, P.-T., Yieh, Y.-H., Tang, K.-L. & Hsing, C.-Y. (1966) *Sci. Sin.* **15**, 544–561
- McCann, J., Choi, E., Yamasaki, E. & Ames, B. N. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5135–5139
- Nesvadba, H. & Roth, H. (1967) *Monatsh. Chem.* **98**, 1432–1436
- Rees, A. R. & Offord, R. E. (1976a) *Biochem. J.* **159**, 467–479
- Rees, A. R. & Offord, R. E. (1976b) *Biochem. J.* **159**, 487–493
- Rüegg, U. T. & Gattner, H.-G. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 1527–1533
- Rüegg, U. T. & Rudinger, J. (1974a) *Isr. J. Chem.* **12**, 391–401
- Rüegg, U. T. & Rudinger, J. (1974b) *Int. J. Pept. Protein Res.* **6**, 447–456

- Rüegg, U. T. & Rudinger, J. (1977a) *Methods Enzymol.* **47**, 111–116
- Rüegg, U. T. & Rudinger, J. (1977b) *Methods Enzymol.* **47**, 116–122
- Rüegg, U. T., Jarvis, D. & Rudinger, J. (1979) *Biochem. J.* **179**, 119–126
- Sakakibara, S., Shimonishi, Y., Okado, M. & Kishida, Y. (1967) *Proc. Eur. Peptide Symp. 8th* 44–49
- Shimonishi, Y., Zahn, H. & Puls, W. (1969) *Z. Naturforsch. Teil B* **24**, 422–427
- Sifferd, R. H. & du Vigneaud, V. (1935) *J. Biol. Chem.* **108**, 753–761
- Spackman, D. H. (1967) *Methods Enzymol.* **11**, 3–15
- Tsou, C.-L., Du, Y.-C. & Xü, G.-J. (1961) *Sci. Sin.* **10**, 332–343
- Weinert, M., Brandenburg, D. & Zahn, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1556–1562
- Weinert, M., Kircher, K., Brandenburg, D. & Zahn, H. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 719–724
- Weygand, F. & Zumach, G. (1962) *Z. Naturforsch. Teil B* **17**, 807–810