The Preparation of Protected Fragments of Lysozyme for Semisynthesis

By A. R. REES and R. E. OFFORD

Laboratory of Molecular Biophysics, Department of Zoology, South Parks Road, Oxford OX1 3PS, U.K.

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This paper reports the development of methods for preparing tryptic fragments of hen's-egg lysozyme in an appropriate state of protection for use in the chemical synthesis of modified polypeptides. 1. We describe the cleavage of the disulphide bridges of the enzyme and the simultaneous protection of the liberated thiol groups by *S*-sulphonation. Lysozyme resisted the usual conditions for this reaction. We have confirmed the stability of the *S*-sulphonyl group to the conditions met in peptide synthesis. 2. We describe the reversible protection of the amino groups of the enzyme by reaction with various anhydrides of 1,2-dicarboxylic acids. We conclude that 2-methylmaleic anhydride and *exo-cis*-3,6-endoxo- Δ^4 -tetrahydrophthalic anhydride are unsuitable for our purpose but that maleic anhydride can, in spite of certain drawbacks, be used. 3. We describe the tryptic cleavage of the thiol- and amino-protected protein and the separation of the fragments. 4. We describe the reversible protection of the carboxylic acid groups (including the specific deprotection of the α -carboxyl group), the imidazolyl group and the α -amino groups of the fragments. Several alternative groups have been evaluated for most of these purposes. The side-chain amides did not present any serious problem of lability. 5. We describe experiments on the stability of the side chain of tryptophan, both protected by formylation and unprotected, to the acid conditions needed for the deprotection of the other functional groups in the peptide. We conclude that protection of tryptophan is unnecessary. We suggest that most of the methods described are of general application in peptide semisynthesis by fragment condensation. An Appendix is included to which points 6-11 appertain. 6. We compare the use of a number of diazoalkanes as mild quantitative agents for the reversible protection of the carboxyl groups of polypeptides. The diazoalkanes were diazomethane, 1-diazo-2,2-dimethylpropane, phenyldiazomethane, diphenyldiazomethane, *p*-methoxyphenyldiazomethane and (putative) 4-methoxy-3-sulphophenyldiazomethane. We have developed general conditions for the use of these reagents and recommend that amino and imidazolyl groups be protected before esterification. 7. *p*-Methoxyphenyldiazomethane seems most suitable for general use. It produces an ester group which is quite readily labile to acid, but not so rapidly as to exclude the possibility of the selective removal of *t*-butoxycarbonyl protection from amino groups. Phenyldiazomethane is also satisfactory for applications that permit a more acid-stable ester group. 8. 1-Diazo-2,2-dimethylpropane has advantages over *p*-methoxyphenyldiazomethane only when azide couplings are intended. 9. Diphenyldiazomethane is very slow to react and gives a very apolar product. We do not in general recommend it for semisynthetic work. 10. Preliminary experiments with the putative 4-methoxy-3-sulphophenyldiazomethane (which is water-soluble) indicate the possibility of acid-labile protection of carboxyl groups without loss of negative charge. The ester also shows a surprising degree of lability to base. 11. All the diazoalkanes produce an ester which can be selectively removed by trypsin from the α -carboxyl group of fragments with C-terminal arginine. 12. A Supplementary Publication (SUP 50071) (37 pages) includes further details of the *S*-sulphonation reactions, of the separation of the tryptic fragments, of experiments on the stability of the side chain of tryptophan and of the preparation of 1-diazo-2,2-dimethylpropane and *p*-methoxyphenyldiazomethane. The Supplement can be obtained from the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., under the terms given in *Biochem. J.* (1976) 153, 5.

Protein semisynthesis involves the use of fragments of natural proteins as ready-made intermediates for the preparation of molecules of modified sequence. A number of semisynthetic proteins have now been made in which natural and synthetic parts have been covalently linked by peptide bonds. The purposes for which these have been made include isotopic tracer work (Borras & Offord, 1970; Halban & Offord, 1975) and nuclear-magnetic-resonance spectroscopy (e.g. Saunders & Offord, 1972; Chaiken *et al.*, 1973; Garner & Gurd, 1975).

Most semisyntheses so far reported have been of the stepwise, as opposed to the fragment-condensation, type. The use of these two terms in protein semisynthesis corresponds to that in the terminology of conventional synthesis, from which they are derived. In stepwise semisynthesis, as it has so far been practised, a few residues are removed from the *N*-terminus and replaced, with appropriate changes, by the use of amino acid active esters. In contrast, fragment-condensation semisynthesis involves, as an early stage, the cleavage of the natural protein by endolytic agents such as trypsin (Offord, 1969*a*) or CNBr (Offord, 1972).

We report the preparation of a number of tryptic peptides of hen's-egg lysozyme (EC 3.2.1.17) protected in such a way as to make it possible to use them for fragment condensation. The peptides have been used (Offord, 1973; Rees & Offord, 1976) for the preparation of long fragments of the enzyme's sequence, which were required for physical studies on their conformation (A. R. Rees, J. E. Thornton & R. E. Offord, unpublished work).

The stages in the production of the protected fragments are as follows: (1) reduction of the disulphide bridges of the protein with simultaneous conversion of the liberated cysteine residues into the *S*-sulphonato derivative; (2) maleylation of the protein's amino groups; (3) cleavage of the protein derivative by trypsin; (4) separation of the tryptic peptides; (5) reversible protection of the α -amino groups of the peptides; (6) reversible protection of the imidazolyl side chain of the histidine-containing peptide; (7) reversible protection of the carboxyl groups of the peptides; (8) selective deprotection of the α -carboxyl group of each peptide by trypsin.

We report experiments that suggest that the reversible protection of the side chains of tryptophan, a notoriously fragile residue, is not always worth while. Steps 5–8 are not needed if the fragment in question is to have other peptides coupled only at its *N*-terminus, and not at its *C*-terminus.

These operations might seem to involve a considerable effort, but it should be contrasted with that involved in synthesizing the fragments by conventional means. When using natural fragments there is always the risk that they have been inadequately purified, or damaged in purification. But in our

opinion the reliability of the chemical structure of the natural product, particularly if it is long, is at least as good as that expected of synthetic ones. The difficulty of preparing protected natural fragments, in contrast with that in conventional synthesis, does not increase at all sharply with chain length.

Experimental

Materials

Common laboratory reagents were obtained from BDH, Poole, Dorset, U.K., and were Analytical Grade. Cysteine hydrochloride was BDH Biochemical Grade. Lysozyme was that of Worthington Biochemical Corp., twice-crystallized (code LY, obtained from Cambrian Chemicals, Croydon CR9 3QL, Surrey, U.K.). We found it superior in its solubility to material from alternative sources. Trypsin (Worthington; code TRTPCK) and the 1-hydroxy derivative of the Boc-NH-CH(CF₃)* reagent were also obtained from Cambrian Chemicals. Trifluoroethanal (fluoral) was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Dialysis tubing was obtained from Scientific Supplies, London EC1 5EB, U.K.

Methods

Standard methods were used for amino acid analysis, end-group determination, paper chromatography and paper electrophoresis as described by Gonzalez & Offord (1971). Papers were stained as described by Offord (1969*b*) and electrophoretic mobilities interpreted as described by Offord (1966).

S-Sulphonato-lysozyme. Hen's-egg lysozyme was dissolved in 8*M*-urea [filtered through a bed of macerated Whatman 3MM paper and freshly deionized by passage through a mixed-bed (BDH MB3) column] to a concentration of 2 mg/ml. A solution of 1*M*-Tris/HCl, pH 7.5, was added to a final concentration of 0.1*M* in Tris. Anhydrous Na₂SO₃ was dissolved in the solution to a final concentration of 0.3*M*. O₂ was bubbled through the mixture for 20 min, and, if necessary, the pH adjusted to 7.5 with 1*M*-HCl. Cysteine hydrochloride was dissolved in the mixture to a final concentration of 0.16*M*. The solution was then transferred to a microbiological incubator rack and shaken, loosely stoppered, at 37°C for 18 h.

At the end of the reaction the solution was dialysed at 10°C in 18/32 Visking tubing over the course of 2 days against three changes of 20 vol. each of distilled water. The non-diffusible residue was freeze-dried. The extent of reaction was checked by examining a tryptic digest of a sample (see the

* Abbreviations: Boc-NH-CH(CF₃)-, 1-*t*-butoxycarbonylamido-2,2,2-trifluoroethyl-; Cbz-NH-CH(CF₃)-, 1-benzoyloxycarbonylamido-2,2,2-trifluoroethyl-; Boc-, *t*-butoxycarbonyl-; Cbz-, benzoyloxycarbonyl-.

Results section and Supplementary Publication SUP 50071).

Maleylation of *S*-sulphonato-lysozyme. *S*-Sulphonato-lysozyme was dissolved in freshly deionized 8M-urea prepared as above to a concentration of 10mg/ml (5 μ mol of amino groups/ml). Sodium tetraborate (1M), brought to pH8.5 with boric acid, was added to a final sodium concentration of 0.2M. The mixture was cooled to 4°C and stirred magnetically. The temperature was kept below 4°C through the subsequent reaction. Small portions of solid maleic anhydride (recrystallized from chloroform) were added. The pH fell at each addition and was restored to an apparent value of 8.5 [glass electrode, standardized against a urea-free buffer at room temperature (20°C)], with 2M-NaOH. The operation was repeated until a 12-fold molar excess of reagent over amino groups had been added. The solution was stirred for 1 h after the apparent pH had been returned to 8.5 after the last addition of anhydride. The pH did not alter during this time. The solution was dialysed in Visking 18/32 tubing [which had previously had its pore size decreased by exposure to boiling NaHCO₃ (1%, w/v) for precisely 10min] against three changes of 20vol. each of 0.5% (w/v) NH₄HCO₃ at 10°C. The solution was left on the freeze-drier [operating pressure (Pirani gauge) below about 13 Pa (0.1 Torr)] for 48 h, to remove all but the last traces of NH₄HCO₃.

These conditions are those of Butler *et al.* (1969), slightly modified for reasons to be discussed below. They were also used for the reaction of *S*-sulphonato-lysozyme with 2-methylmaleic anhydride (Dixon & Perham, 1968) and *exo-cis*-3,6-endoxo- Δ^4 -tetrahydrophthalic anhydride (Riley & Perham, 1970). The extent of reaction was determined on a sample by the method of Levy & Chung (1955), with reduced carboxymethylated lysozyme as a standard.

Tryptic cleavage. The freeze-dried product of the previous stage was dissolved in 0.5% NH₄HCO₃ to a final concentration of 10mg/ml. The solution was warmed to 37°C. Solid trypsin [treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK)] was dissolved in the solution; one-hundredth of the weight of lysozyme was used. The digestion was stopped after 90 min at 37°C by freeze-drying. The extent of digestion and the degree of non-specific cleavage was determined on a sample by observing the pattern of a paper electrophoresis at pH6.5. We find that the peptide bond between tryptophan-108 and valine-109 is unusually susceptible to chymotrypsin-like activity. The combined effect of such activity and trypsin is the release of the peptide Val-Ala-Trp-Arg. This peptide migrates with a mobility of +0.38 (Asp = -1). It gives a slowly appearing red colour with the Cd/ninhydrin stain and a positive stain for tryptophan. We have, for this study and a previous one (Rees & Offord,

1972), separated and analysed all the significant components of tryptic digests of lysozyme made with a large number of samples of trypsin. Some samples were used both before and after treatment with a specific inhibitor of chymotrypsin. Our experience with these digests, and with the similar examination of digests of two other proteins in the course of sequence determination (Gonzalez & Offord, 1971; Furth *et al.*, 1974) led us to conclude that the appearance of this tetrapeptide in a digest is an unusually sensitive test for non-tryptic cleavage.

Separation of the peptides. Two methods for the bulk separation of the peptides are described in detail in Supplementary Publication SUP 50071. In the first, a column (30cm $\times$ 2.5cm) of Sephadex A-25 cation-exchanger was used, eluted with ammonium formate, in a linear gradient from 0.05M (adjusted to pH8.4 with aq. 18M-NH₃) to 0.7M (adjusted to pH8.0 with aq. 18M-NH₃), of total volume 1.6litre; 2-3g of digest was separated at a time. Peptides M3, M4 and M11 (see Table 2) were obtained from the column in a satisfactory state of purity (see below). Peptide M4 was eluted as a suspension. Peptides M1, M2, M5 and M7 could be obtained pure by gel filtration of the appropriate peaks on a column (90cm $\times$ 2.8cm) of Sephadex G-25 in 0.1M-pyridine. Peptides M6, M8 and M10 did not emerge from the column.

An alternative method of purification involved an initial gel-filtration step on a column (180cm $\times$ 4.2cm) of fine-grade Sephadex G-50 in NH₄HCO₃ (0.5%, w/v); 750mg of digest could be separated at a time. Peptide M6 emerged last from this column, and was electrophoretically pure. The other peptides were separated subsequently by ion-exchange chromatography of the appropriate pooled fractions (Supplementary Publication SUP 50071). Peptide M7 could also be obtained by preparative paper electrophoresis at pH6.5.

The purity of the peptides was assessed by dansyl end-group determination, amino acid analysis and paper electrophoresis. Peptides that gave single end groups, no visible evidence of inhomogeneity when run on paper in at least two systems, and amino acid analyses (Table 2, below) compatible with their known sequences were taken to be in a satisfactory state of purity. (The amino acid composition of peptide M4 was not totally satisfactory, and is discussed below.)

Reversible protection of the α -amino group of the peptides. Benzyloxycarbonylation was carried out as described by Offord (1969a). The alternative, *t*-butoxycarbonylation, was applied as follows. The peptide was dissolved in dimethyl sulphoxide (redistilled) to a concentration of 50 μ mol/ml. All subsequent operations were carried out under a hood. *t*-Butoxycarbonylazide was added, usually in a 100-fold excess over amino groups. The mixture was stirred magnetically during the addition to avoid

precipitation of the peptide by the azide. *N*-Ethylmorpholine (redistilled) was added until the pH, as indicated by the use of BDH narrow-range pH papers, was about 9. The ranges used were 6–8 and 8–10, of which the former is the most informative. The papers were first moistened with a small drop of water. When the drop had soaked into the paper, 0.1–0.5 μ l of peptide solution was discharged from a microcapillary tube on to a point just inside the wet zone. When carried out in this way, the method gave consistent results and success of this and other reactions in non-aqueous media correlated with our having first obtained the appropriate colour. We do not regard the method as a means of obtaining an accurate estimate of $[H^+]$, but merely as a means of process control.

When the indicated pH was satisfactory, the mixture was stirred at room temperature until the reaction was found, by electrophoresis of a sample, to be complete. Peptide M3 appeared to be protected on its histidine side chain also (see below). The reaction time was usually 24 h, but peptide M2 in particular took 48–72 h, even with a 400-fold excess of azide. The reaction mixture was freeze-dried exactly as though it was an aqueous solution. The residue was extracted with ether to remove excess of reagent and side product.

N-Ethylmorpholine was preferred as a base to triethylamine as it is much more easy to overshoot the desired apparent pH when the latter is used, and there is a much greater tendency for the indicated pH to wander during a reaction or even on standing. Dioxan/water (Schnabel, 1967) and pyridine were less satisfactory as solvents (Rees, 1974). Dimethylformamide was adequate for all but the most insoluble peptides (such as M2), when the use of dimethyl sulphoxide was essential.

The *N*-terminal peptide of lysozyme (M1) was obtained from the digest with both its α - and ϵ -amino groups maleylated. These were freed by incubating the maleyl-peptide in acetic acid/pyridine/water (10:1:89, by vol.), pH 3.5, for 100 h at 37°C with occasional shaking. The deprotection was followed electrophoretically and by the dansyl end-group method (Gray, 1967). The α,ϵ -di-Boc derivative was then prepared by the general method given above.

Protection of the histidine side chain of peptide M3. Peptide M3 emerged from the previous step with the Boc group on both its α -amino group and its side chain. The side-chain Boc group is stable to some of the acid media that will deprotect the α -amino group (Fridkin & Goren, 1971) and might therefore seem to be a suitable form of protection in itself. Unfortunately, it is too readily deprotected in the presence of basic, or nucleophilic, reagents (Losse & Krychowski, 1970; Schnabel *et al.*, 1971; Rees, 1974) to serve the present purpose and must be removed. This was done by suspending the di-

protected product in 2M-acetic acid at 0°C for 1 h, at a peptide concentration of 10 μ mol/ml. The solution was freeze-dried immediately. Electrophoretic tests (see Table 3) and staining with ninhydrin showed that the side-chain protection had been removed, but that the α -amino protection (and the peptide's side-chain amide group) remained.

Protection of the histidine side chain with N-benzyl-oxy-carbonyl-1-chloro-2,2,2-trifluoroethylamine. The reagent was prepared from benzylcarbamate and trifluoroethanal as described by Weygand *et al.* (1966). The conditions for reaction that Weygand *et al.* (1967a) used for amino-protected histidine were followed, with modifications to take into account the lower reactivity of the side chain in the peptide, and the peptide's insolubility in the recommended solvent. The [α -Boc-histidine]peptide M3 was dissolved at a concentration of 10 μ mol/ml in dimethyl sulphoxide and cooled to 4°C. Meanwhile a solution was prepared containing a 30-fold excess of the protecting reagent in 0.25 ml of dimethyl sulphoxide, adjusted with *N*-ethylmorpholine to an indicated pH (obtained with pH paper as described above) of between 8 and 9. White fumes were observed on the addition of base, which were probably due to traces of acid substances left over from the preparation of the reagent. The reagent solution was cooled to 4°C. The peptide and reagent solutions were mixed, and a quantity of *N*-ethylmorpholine was added that was equivalent to the amount of peptide. The reaction was allowed to proceed for 2 h at 4°C, followed by 30 min at 10–15°C. The mixture was freeze-dried and extracted with ether to remove unwanted materials. The degree of completion of the reaction was assessed by paper electrophoresis.

Protection of the histidine side chain with N-Boc-1-trifluoroacetoxy-2,2,2-trifluoroethylamine. We attempted to prepare the precursor of this reagent (the free 1-hydroxy compound) by the method of Weygand *et al.* (1967b). Even after repeated attempts the method failed, in our hands, to give any product. We suspect that the trifluoroethanal used in the reaction polymerizes under the conditions as published. The 1-hydroxy derivative was therefore purchased and converted into the trifluoroacetyl derivative by the method of Weygand *et al.* (1967a). Some 4.35 ml of a solution of 0.35 ml of trifluoroacetic anhydride in 7.5 ml of ice-cold redistilled pyridine was added, at 0°C, to 323 mg (1.5 mmol) of the 1-hydroxy compound. The solution, which turned pale-brown during the addition, was left at 25°C for 30 min. [α -Boc-histidine]peptide M3 was then added (150 μ mol) as a saturated solution in redistilled pyridine, and the mixture stirred for 24 h. If an electrophoretic check showed that reaction was not complete (Table 3) a further quantity of reagent (750 μ mol) was made and allowed to react for another 24 h. This two-step procedure was found to be better than a single

application of 2.25 mmol of reagent. When reaction was complete the mixture was taken to dryness on a rotary evaporator and extracted with ether to remove unwanted materials.

Reversible protection of carboxyl groups. The method was based on that of Offord (1969a). The esterifying agents most frequently used were phenyldiazomethane and *p*-methoxyphenyldiazomethane in a cumulative 50–100-fold excess over carboxyl groups. 1-Diazo-2,2-dimethylpropane was also used in some instances. The solvent system was usually ethanol/water (17:3, v/v) or dimethylformamide/water (17:3, v/v) controlled by an autotitrator with aq. dilute HCl to an apparent pH (glass electrode) of between 4 and 5. Reaction times at 0°C ranged from 20 min to 2 h. Full details of the use of diazoalkanes are given in the Appendix.

Selective deprotection of α -carboxyl groups by trypsin. The method used was that of Offord (1969a) with minor modifications. We found that dimethylformamide/water (1:1, v/v) was the most generally useful solvent mixture. Trials with pyridine/water mixtures showed that, as the proportion of water was decreased below 100%, a point came, which differed from peptide to peptide, at which trypsin ceased to act. Rees (1974) found that the addition of Ca^{2+} to a final concentration of 0.05 M allowed one to decrease the amount of water to a lower value than would otherwise be the case, but even so digestions seldom proceed at a satisfactory rate below 50% water. We have the subjective impression that dimethylformamide/water mixtures permit the expression of the esterase activity of trypsin at lower concentrations of water than do pyridine/water mixtures and are also better solvents for long protected peptides. If Ca^{2+} is present the amount of water can be decreased to 30% for many peptides but below that the tryptic activity is negligible in our hands. A few experiments suggested that dimethyl sulphoxide/water mixtures of a similar range of compositions would be even better solvents for the peptides and would permit much the same measure of tryptic activity. (The drying down of aqueous dimethyl sulphoxide is made difficult by frothing.) Proteolysis requires, for comparable long-chain substrates, much more enzyme and an even higher ratio of water to the chosen organic solvent in order to proceed at the same rate as esterolysis (see the Appendix).

All the peptides in the present study were specifically deprotected by the following procedure. Protected peptide was dissolved in dimethylformamide/water (1:1, v/v) to a concentration of up to 10 mM and titrated to an apparent pH of 7 (glass electrode) with 0.05 M-NaOH. (This value is below that normally used for trypsin but was found to be necessary to avoid loss of side-chain amide in peptide M6.) The base was added slowly, with

vigorous stirring, to avoid high local concentrations of NaOH. A solution of trypsin was made up in the same solvent at a concentration of 5 mg/ml. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (1 M) was added to a final concentration of 0.05 M. The apparent pH was adjusted to 7. Then 20 μl of the trypsin solution was added to the peptide solution, and the ensuing reaction followed by observing the extent of base uptake (usually 95–105% of the theoretical value for the liberation of one carboxyl group). The reaction mixture was freeze-dried when the theoretical amount of base had been taken up (from a few minutes to 2 h, depending on the peptide). The peptide material was freed from trypsin and salts by gel filtration on lipophilic Sephadex LH-20 in dimethylformamide, ethanol, ethanol/water (17:3, v/v) or dimethylformamide/water (17:3, v/v), depending on the solubility of the peptide derivative. A column of 105 cm $\times$ 2.2 cm was usually satisfactory for the purification of 50 mg of peptide. The protected derivative of M6 peptide emerged too late on the column to be separable from the by-products of esterification (see the Appendix). These were partly removed by extraction of the protected peptide into pyridine/water (1:1, v/v). The last traces could only be removed from the product after coupling, when the increased molecular size made it possible to use gel filtration.

A final check on the state of the α -carboxyl group was made by observing the electrophoretic mobility of samples of those peptides that would still run in the protected state and by the ability of carboxypeptidase B (in 50 μl of 0.2 M-N-ethylmorpholine/acetate buffer, pH 8, 20–30% in pyridine, digested for 2 h at 37°C with an enzyme/substrate ratio 1:100, w/w) to liberate arginine from samples of the peptide after treatment.

Preliminary experiments with trypsin bound to Sepharose beads showed that the removal of α -esters proceeded at much the same rate as that brought about by the same number of units of free enzyme. The tryptic activity could be removed at the end of the digest by centrifugation and washing without appreciable loss of peptide.

General deprotection of the peptides. The Boc-, Boc-NH-CH(CF_3)- and *t*-amyl-groups were removed by dissolving the protected peptides in anhydrous trifluoroacetic acid (redistilled) at a concentration of about 10 mg/ml and leaving for 40–60 min at 4°C. The *p*-methoxybenzyl group was found in pilot experiments to require 40 min at 4°C for deprotection of the side-chain carboxyl group of glutamic acid in peptides and the α -carboxyl group of peptides, 120 min at 4°C for the side-chain carboxyl group of aspartic acid in peptides, and 300 min at 4°C for the free carboxyl group of an ϵ -maleyl-lysine residue in peptides. The α,ϵ -diester of bismaleyl-lysine in peptide M1 was extremely slow to deprotect, which is the reason for the demaleylation of this peptide and

the protection of its amino groups by the Boc group, as described above. At the end of the reaction it was found best, if at all possible, either to precipitate the peptide from the trifluoroacetic acid by adding several volumes of cold diethyl ether (peroxide-free, dried over sodium) or to add 100 vol. of cold water, with cooling and stirring, and then to freeze-dry. Although anhydrous trifluoroacetic acid could usually be removed *in vacuo* without the slightest difficulty, the last traces occasionally and inexplicably lingered, leaving the sample as a yellow strongly acidic oil.

The benzyl-, Cbz- and Cbz-NH-CH(CF₃)- groups were removed by bubbling purified HBr through a solution of the peptide in anhydrous trifluoroacetic acid (redistilled) for 60 min at 0°C, as described by Stewart & Young (1969).

Exposure to HF (which removes all the above protecting groups) was for 60 min at 0°C as described by Stewart & Young (1969). A CoF₃ trap was used. The maleyl group was removed as described above for peptide M₁, and the sulphonato group as shown in Table 1.

Experiments on the need for protection of the side chain of tryptophan. These are described in detail in Supplementary Publication SUP 50071. Boc-tryptophan and Boc-tryptophan benzyl ester (the latter was made by the methods described in the Appendix) were subjected to various acidic treatments both with and without scavengers. The degree of damage to the tryptophan was assessed by the amino acid analyser, by u.v. spectroscopy and in certain cases by mass spectrometry. The spectroscopic means of following the acid deprotection of Boc-tryptophan depends empirically on the observed difference in spectrum between Boc-tryptophan and tryptophan when in acid solution (see Supplementary Publication SUP 50071).

Results

Extent of *S*-sulphonation

Our conditions are based on those of Chan (1968). We found that his method, and that of Swan (1957) did not take the reaction to completion when applied to lysozyme, as judged by paper electrophoresis of a tryptic digest (see Supplementary Publication SUP 50071). Material produced as described in the Experimental section gave a clean pattern of peptides on electrophoresis at pH 6.5, which closely resembled that of totally reduced and carboxymethylated lysozyme. The other procedures gave products that showed no distinct cationic peptides. We did not obtain a quantitative estimate of the extent of reaction with our conditions (although it appears to be complete) since our only object was to obtain a reasonable yield of the correct tryptic fragments.

Stability of the *S*-sulphonato group

This group has been advocated for conventional peptide synthesis (e.g. Hofmann & Katsoyannis, 1963), but is not much used. One reason is probably that its polarity decreases solubility in the apolar organic solvents traditionally used in peptide synthesis. This is less of a drawback in semisynthesis, where it has been found best to keep the solubility of the fragments in polar solvents as high as possible. Another potential difficulty, the possible screening of the α -amino group by interaction with the negatively charged side chain, would be less often a problem when it is a peptide that is protected (but see the protection of peptide M₂ in the Experimental section). Another possible drawback, that the group might not be stable to conditions encountered in semisynthetic work, would be more serious if

Table 1. *Studies on the stability of the S-sulphonato-group*

For details of treatments, see the Experimental section.

Reagent	Duration of treatment	Temperature (°C)	Peptide used	Electrophoretic mobility at pH 6.5*		
				Observed	Predicted	
					as -S-SO ₃ ⁻	as -SH
Trifluoroacetic acid (anhydrous)	2 h	0	M11	0	0	0.55
Hydrazine (anhydrous)	30 min	25	M11	0	0	0.55
0.05 M-NH ₃	18 h	25	M11	0	0	0.55
Benzylation with phenyldiazomethane followed by deprotection in HBr/trifluoroacetic acid	See the Experimental section		M11	0	0	0.55
2-Mercaptoethanol/pyridine/water (1:5:5, by vol.)	1 h	25	Boc-M2	-0.44	-0.67	-0.44
2-Mercaptoethanol/0.01 M-NH ₃ (1:10, v/v)	1 h	25	M2	-0.22	-0.48	-0.25

* Mobility relative to aspartate -1.

confirmed. Model experiments were therefore carried out on some of the *S*-sulphonato-peptides (Table 1). It appears that the group is quite stable enough for general use, but that it can be readily removed when required.

Acylation of amino groups

The method of Levy & Chung (1955) (see the Experimental section) did not detect any free amino groups after maleylation. Semiquantitative estimates of the extent of reaction with the other anhydrides did not suggest that there were any significant differences in efficiency.

We chose to use less reagent than did some of the authors cited in the Experimental section because we found that too large an excess led to there being spurious peptides in the tryptic digest. The spurious peptides were also observed more readily if the acylation was conducted at room temperature. These peptides suggested that there might have been acylation of side-chain hydroxyl groups, a danger to which Gibbons & Perham (1970) drew attention.

Studies on the electrophoretic mobility of samples shows that the maleyl group is completely resistant to long exposure to HF and trifluoroacetic acid.

The methylmaleyl and the bridged tetrahydrophthaloyl derivative should be more useful than the maleyl group because they are more readily removed. The half-times for removal of the three groups in aqueous solution are 1–1.7 h in 40 mM-HCl at 20°C for the 2-methylmaleyl group (Dixon & Perham, 1968), 4–5 h at pH 3.5 at 25°C for the bridged tetrahydrophthaloyl group (Riley & Perham, 1970) and 11 h at pH 3.5 at 37°C for the maleyl group (Butler *et al.*, 1969). Possibly because of the shorter exposure to acid that is required, deprotection restored full enzymic activity to lysozyme that had been acylated with the first two reagents (Habeeb & Atassi, 1970; Riley & Perham, 1970), whereas only 83% of the activity was restored on deacylation of maleyl-lysozyme (Habeeb & Atassi, 1970). However, an extensive series of comparisons (Rees, 1974) showed that only maleic anhydride could be used for the present study. Certain of our methylmaleyl-peptides appear to be deacylated on handling even at neutral pH, or when stored frozen in neutral solution; for instance peptide M10 ($m = -0.4$) had been converted into a neutral peptide after being freeze-dried from 0.5% NH_4HCO_3 , stored dry at -20°C for 4 weeks and redissolved in the original solvent [compare with Habeeb & Atassi (1970)]. Lysine residues that are next to negatively charged groups may be particularly susceptible to deacylation as a result of more ready protonation of the free carboxyl group of the methylmaleyl substituent under such circumstances (Rees, 1974).

The useful extra degree of stability of the bridged tetrahydrophthaloyl group was outweighed by the

Fig. 1. Double forms of peptides M2 and M4

(a) Result of chromatography on Sephadex A-25 of a pooled fraction obtained from a Sephadex G-50 run of a tryptic digest of sulphonated lysozyme acylated with *exo-cis*-3,6-endoxo- Δ^4 -tetrahydrophthalic anhydride. The column systems are described in the Experimental section and full details are given in Supplementary Publication SUP 50071. Each tube contained 9 ml, and the molarities of eluates written above the peaks were estimated by conductivity measurements. (b) Electrophoretograms (pH 6.5) of material from the peaks. The mobility scale is relative to Asp = -1. The neutral point has moved from the origin by endosmosis. The colours (yellow and orange) are those obtained on staining with Cd/ninhydrin and are the same as those given by the maleylated forms. The peptides were identified by preparative electrophoresis and amino acid analysis.

disadvantage [to which Riley & Perham (1970) drew attention] that the acyl-peptides might be formed as a mixture of diastereo isomers. If, as Riley & Perham (1970) hoped, the properties of the diastereo isomers were not very different, their existence could be ignored. However, peptides M2 and M4 both appeared as doublets separable on ion-exchange chromatography. By chance one of each of the forms of the M2 peptide travels with one of each of the forms of peptide M4 (Fig. 1). In spite of the differences in length, peptides M2 and M4 cannot easily be separated by gel filtration (peptide M4 is the longer, but also the more aromatic). The problem of separation was aggravated by the considerable insolubility and difficulty in handling of all forms of peptide M4 in most systems. The use of the bridged tetrahydrophthalic anhydride (which was the only one to produce the doubling phenomenon) was therefore abandoned.

Protection of the histidine side chain

Chillemi & Merrifield (1969) reported that the Cbz-NH-CH(CF₃)-group was inadequately stable to the lengthy cumulative exposure to trifluoroacetic acid experienced in stepwise synthesis of very long peptides. This should not be taken to mean that it is insufficiently stable for use in stepwise syntheses of short peptides and in fragment-condensation work. We find that several hours treatment in trifluoroacetic acid produces no visible deprotected product. Some 0.0126 μ mol of histidine was recovered from a sample of 0.025 μ mol of α -Boc-N^π-Cbz-NH-CH(CF₃)-histidine that was treated with 6M-HCl for 24h *in vacuo* at 108°C. Some 0.023 μ mol of histidine was recovered when a similar sample of the protected amino acid was treated with anhydrous HF for 1h at 0°C. We conclude that the Cbz-NH-CH(CF₃)-group is extremely resistant to all but the strongest of acid conditions and constitutes a useful form of protection.

We found by electrophoresis that significant deprotection of the N^π-Boc group of [α -di-Boc-histidine]peptide M3 occurred at an apparent pH4.5 (glass electrode) in ethanol/water (17:3, v/v) after 20min at 0°C. These are the conditions used for esterification of the carboxyl groups, and so the protection of the imidazole by the Boc group could not be used. The Boc-NH-CH(CF₃)- group proved to be perfectly stable until totally removed by anhydrous trifluoroacetic acid as described in the Experimental section.

Characterization of the fragments

Table 2 gives end groups and amino-acid compositions for the fragments isolated. The mobilities are given in Table 3. Fig. 2 shows their sequence and indicates the functional groups in need of protection. M9, a dipeptide, was discarded, as was the C-terminal fragment of the enzyme, free leucine.

The amino acid analyses serve to establish that the peptides have been correctly identified, and, in general, they give no indication of any serious cross-contamination. Only peptide M4 contains significant quantities of an amino acid (isoleucine) not accounted for in its sequence. The value for arginine for peptide M4 is also rather high. The isoleucine is also found in the compositions of the longer fragments made from peptide M4 (Rees & Offord, 1976) and thus illustrates an intrinsic difficulty likely to be encountered from time to time in semisynthetic work. The cause must presumably be either cross-contamination or a genetically based microheterogeneity. We are reluctant to propose the latter explanation without direct evidence, although we are unable to find any isoleucine-containing sequence in the published structure of lysozyme that would have the observed

effect on the composition and which could reasonably be proposed as a cross-contaminant. Like all the other peptides, M4 has no visible electrophoretic impurities and gives a single end group. Equally, tryptic digests of peptide M4 after deprotection of the lysine give only the two expected peptide spots on electrophoresis and chromatography. We are also surprised that a completely unrelated cross-contaminant should have followed the semisynthetic products made from peptide M4 through the couplings and the subsequent purification schemes.

Characterization of the fragments after subsequent protection

Table 3 gives the characteristics of the fragments in the various stages of protection. The solubility and electrophoretic behaviour of the protected peptides were closely similar whichever of the three ester groups (benzyl-, *p*-methoxybenzyl- or *t*-amyl-) was used. The study of a few examples suggested that methylated peptides tended to be more soluble and are electrophoresed more easily. Peptides M3, M4, M5, M6 and M8 contain side-chain amides, and some of those in peptides M5 and M6 are known (Jollès *et al.*, 1963; Canfield, 1963; Rees & Offord, 1972) to be labile to the conditions encountered in the determination of the amino acid sequence. Electrophoretic observation (Table 3) suggests that an amide group in peptide M5 has been partly lost even by the time the fragment had been isolated from the digest. The existence of such labile amide groups is an inherent disadvantage of the semisynthetic approach, although it is not completely absent from conventional peptide synthesis. In the present instance, the loss is only partial, and could probably be lessened by taking advantage of the fact that peptide M5 has no lysine or cysteine residues needing protection. The peptide could therefore be obtained from a digest of protein that had received far less handling. Electrophoretic examination showed that the side-chain amide of peptide M6 was lost to a significant extent (much more than if unprotected peptide was similarly treated) if the selective deprotection of the esterified form by trypsin took place at pH8.5. At pH7 (see the Experimental section) there were no visible losses. There was no evidence for loss of any of the other side-chain amides at any stage of isolation, protection or deprotection.

Samples of some of the peptides that were more highly protected with the Boc-, Boc-NH-CH(CF₃)- and *p*-methoxybenzyl groups were treated with trifluoroacetic acid and then with pH3.5 buffer (see the Experimental section). They were then subjected to paper electrophoresis at pH6.5. The papers were stained with Cd/ninhydrin, and overstained for ninhydrin-negative material with Cl₂. Where appropriate, duplicate runs were overstained for tryptophan, tyrosine or histidine. Only peptide M3 gave a

Table 2. *Characterization of the tryptic fragments*

Predicted ratios are given in parentheses. Other amino acids were not usually seen in readily quantifiable amounts and in any case at levels less than 0.1 residue. The predicted and observed mobilities of the fragments are given in Table 3. + indicates that the residue was present but not quantified. No other α -amino groups were detected than those shown. The results are all in agreement with the known sequence. The analysis of peptide M8 was carried out by Dr. W. H. Johnson. The peptide Gly-Cys-Arg-Leu was also found with peptide M11 in some digests. Leucine is the C-terminal residue of the protein.

Protein	SO ₃ H										
	Amino acid composition (molar ratios)										
	M1	M2	M3	M4	M5	M6	M7	M8	M10	M11	
Aspartic acid			2.00 (2)	3.74 (4)	4.20 (4)	2.00 (2)		7.0 (7)	1.0 (1)		
Threonine				1.89 (2)	1.90 (2)		0.95 (1)	1.1 (1)	0.9 (1)		
Serine				1.83 (2)	1.74 (2)		0.92 (1)	4.8 (5)			
Glutamic acid		1.10 (1)		2.00 (2)	1.14 (1)				1.0 (1)		
Proline							0.95 (1)	0.9 (1)			
Glycine	1.20 (1)		1.11 (1)	2.09 (2)	1.98 (2)	0.98 (1)	1.00 (1)	2.0 (2)	1.0 (1)	1.16 (1)	
Alanine		3.00 (3)		2.98 (3)				4.9 (5)	1.0 (1)		
Cysteine		+		+		+		+		+	
Valine	0.96 (1)	(1)		(1)		(1)		(3)		(1)	
Methionine		0.95 (1)						0.8 (1)			
Isoleucine				0.24 (0)	1.57 (2)			2.4 (3)	0.7 (1)		
Leucine		1.10 (1)	1.00 (1)	1.00 (1)	0.95 (1)			3.0 (3)			
Tyrosine			0.95 (1)	0.90 (1)	0.94 (1)						
Phenylalanine	1.00 (1)			2.07 (2)							
Tryptophan				+		+		+	+		
Histidine			0.92 (1)	(1)		(2)		(2)	(1)		
Lysine	0.98 (1)	1.00 (1)		0.93 (1)				1.8 (2)	0.8 (1)		
Arginine	1.11 (1)	1.16 (1)	0.98 (1)	1.20 (1)	1.00 (1)	0.81 (1)	1.11 (1)	0.9 (1)	0.7 (1)	1.00 (1)	
End group	Lys*	Cys†	His‡	Gly	Asn§	Trp	Thr	Asn§	Cys¶	Gly	

* Found as bis-Dns-Lys on a demaleylated sample.

† Found as Dns-Cys(SO₃H), which runs with Dns-Cys(O₃H).

‡ Found as a mixture of the α - and bis-Dns compounds.

§ Found as Dns-Asp.

|| No dansylated compound found. Enzyme digest of the Boc-peptide gave 1.1 residues of tryptophan.

¶ Determined on a sample obtained from reduced and carboxymethylated lysozyme.

spot other than that corresponding to the original unmodified peptide. This spot, which was minor and often absent, corresponded in mobility to the monoester of the peptide, suggesting that the time of acid exposure for the removal of the ester groups (see the Experimental section) was barely adequate.

Experiments on the stability of the indole side chain

We report in Supplementary Publication (SUP 50071) the results of exposing Boc-tryptophan and Boc-tryptophan benzyl ester to a number of acid conditions [anhydrous trifluoroacetic acid, acetic acid 2M in HCl (anhydrous), HBr in tetranitro-

Fig. 2. Amino acid sequences of the peptides

The groups in need of protection are indicated. The amino acid sequences are due to Jollès *et al.* (1963) and Canfield (1963). Differences between these two reports were reinvestigated by Rees & Offord (1972). im, Unprotected imidazole side chain.

methane, trifluoroacetic acid under N_2 , trifluoroacetic acid/water (19:1, v/v) at a variety of temperatures (between 0° and 20°C) with or without the use of scavengers, such as Cbz-methionine and tryptamine, all in tenfold excess. We have also

compared the effect of isolating the deprotected tryptophan from the acid by drying-down the mixture with the effect of isolating by dilution into an appropriate aqueous buffer. We found that for the sort of acid exposure required for the deprotection

Table 3. *Mobilities of fragments in the various stages of protection*

The mobilities are not significantly affected by the nature of the ester group (benzyl-, *p*-methoxybenzyl- and, in those cases where values were determined, *t*-amyl-) or (except for decreased stability) by the substitution of methylmaleyl for maleyl. All cysteine residues were present as Cys(SO₃H) throughout. Measurements and predictions of mobility at pH 6.5 were as described by Offord (1966).

Peptide	Assumed state of protection					Mobility at pH 6.5		R_F in butanol/acetic acid/water/pyridine (30:6:24:20, by vol.)
	α -NH ₂	ϵ -NH ₂	α -CO ₂ H	Side-chain CO ₂ H	Side-chain imidazole	Observed	Predicted	
M1	Maleyl	Maleyl	Free	—	—	-0.63	-0.65	—
	Free	Free	Free	—	—	0.9	0.9	—
	Boc	Boc	Free	—	—	0	0	0.32
M2	Free	Maleyl	Free	Free	—	-0.47	-0.44	—
	Boc	Maleyl	Free	Free	—	-0.68	-0.64	—
	Boc	Maleyl-ester	Ester	Ester	—	Does not run	—	0.9-1.0
	Boc	Maleyl-ester	Free	Ester	—	Not determined*	—	0.9-1.0
M3	Free	—	Free	Free	Free	0.07	—	0.35
	Boc	—	Free	Free	Boc-NH-CH(CF ₃)-	-0.25	-0.28	—
	Boc	—	Ester	Ester	Boc-NH-CH(CF ₃)-	Insoluble	—	0.97
	Boc	—	Free	Ester	Boc-NH-CH(CF ₃)-	0	0	0.8
M5	Free	—	Free	Free	—	-0.16	-0.17	—
						(-0.35)†	(-0.34)	—
	Boc	—	Free	Free	—	-0.35	-0.32	—
						(-0.5)†	(-0.52)	—
	Boc	—	Ester	Ester	—	Does not run	—	—
	Boc	—	Free	Ester	—	Not determined‡	—	—
M6	Free	—	Free	Free	—	-0.25	-0.26	—
						(-0.45)†	(-0.51)	—
	Boc	—	Free	Free	—	-0.42	-0.38	—
						(-0.65)†	(-0.72)	—
	Boc	—	Ester	Ester	—	Does not run	0	—
						(-0.22)†§	(-0.22)	—
	Boc	—	Free	Ester	—	-0.22	-0.22	—
M7	Free	—	Free	—	—	(-0.42)†§	(-0.44)	—
	Free	—	Free	—	—	0.46	0.46	0.3
	Boc	—	Free	—	—	0	0	0.5
M10	Free	Maleyl	Free	Free	—	-0.42	-0.45	—
	Boc	Maleyl	Free	Free	—	-0.61	-0.65	0.75
	Boc	Maleyl-ester	Ester	Ester	—	Does not run	—	1.0
	Boc	Maleyl-ester	Free	Ester	—	Does not run‡	—	—
M11	Free	—	Free	—	—	0	0	—

* The deprotection of the α -CO₂H was established by the successful use of the peptide in coupling reactions (A. R. Rees & R. E. Offord, unpublished work).

† Small quantities of what was taken to be the des-amido form (see the text). The des-amido forms of peptides M5 and M6 were obtained separately from their parent peptides during the bulk separations and discarded.

‡ The deprotection of the α -CO₂H was established by carboxypeptidase B digestion, qualitatively for peptide M5 and quantitatively for peptide M10.

§ Obtained only on prolonged handling of the derivative.

|| Trailed.

of groups that are a little less acid-labile than the Boc group (say 60min in anhydrous trifluoroacetic acid at 20°C) the best results were obtained by the simplest procedure. We recovered 78% of the tryptophan simply on drying down. All other conditions, including those in which scavengers were used, gave recoveries ranging from a few percent to over 20% lower, except for HCl (2M) in acetic acid for

40min at 10°C plus 40min at 20°C which gave 80% recovery whether dried down or isolated by dilution into buffer.

Alakhov *et al.* (1970) observed a mass-spectrometric peak, *m/e* 56, from samples of Boc-tryptophylglycine methyl ester that had been deprotected with trifluoroacetic acid at 20°C for 60min. They concluded that butylation of the indole ring had taken place

on deprotection. Although we observed mass-spectrometric peaks corresponding to both the indole and benzyl moieties of a sample of Boc-tryptophan benzyl ester that had been deprotected under these conditions, we did not observe the peaks at m/e 56. We conclude that the butylation side reaction does not always occur, even under these rather severe conditions.

When we followed the acid deprotection of Boc-tryptophan in the spectrophotometer at 278 nm (see Supplementary Publication SUP 50071), we found that the maximum percentage of free tryptophan (96%) was obtained after 17 min at 4°C. This exposure is too mild for the removal of many protecting groups.

We investigated the protection of the indole ring by formylation, by the method of Previero *et al.* (1967). We deprotected Boc-histidine in the presence of an equivalent quantity of tryptophan, with and without protection by formylation. Using trifluoroacetic acid at 4°C for 1 h, we recovered 89% of the tryptophan without formylation and between 81 and 84% with formylation. We concluded that formylation did not show any clear advantages for our type of work.

Discussion

The peptides, prepared as above, need only the removal of traces of free amino compounds and carboxylic acids to be ready for coupling. The loss of side-chain amides, which might be expected to be a major drawback of semisynthesis as opposed to conventional synthesis, has not, on the evidence that we have, proved to be a problem. Doubtless, amides will be found in some proteins that are too labile to survive our techniques. Those undertaking conventional syntheses can meet this problem (though they rarely choose to do so) by inserting the amide-containing residues in a protected form [for example, Akabori *et al.* (1961) and Shimonishi *et al.* (1962)]. This cannot guard against amide loss on or after deprotection. Should the loss in fact occur the conventional synthesis, like its semisynthetic analogue, could only be salvaged if the deamidated protein has satisfactory biological activity. Observation of the activity of the partially deamidated forms of natural proteins suggests that this requirement will quite often be met. It would be essential to know at which sites and to what extent deamidation had occurred, and the particular methods of scrutiny used in the present work would be suitable and easy to apply.

We have not protected the side chains of arginine, of the hydroxy amino acids, of asparagine and glutamine and of methionine because, although these are sometimes protected in conventional syntheses, many polypeptides have been successfully made with their

side chains free at all stages. We have protected the imidazole side chain more against the alkylating action of the diazoalkanes that we use (Offord, 1969*a*; Hayward & Offord, 1971 and see appendix) than against any other danger. We have not protected the indole ring of tryptophan because we found it unprofitable to do so. However, our experiences with the indole ring suggest to us (as is also the experience of many of those engaged in conventional peptide synthesis) that it is easiest to work on proteins that have little or no tryptophan. For work on proteins rich in tryptophan there is a clear need for protecting groups that do not have to be removed in strong acid.

We feel it necessary to explain our choice of maleic anhydride for the protection of the side-chain amino groups. We had hoped to use one of the other anhydrides because we felt that the advantages of their solubilizing power, resistance to strong acid and above all ease of removal outweigh the apparent oddity of protecting one functional group by introducing another. In the event, as has been described, neither was found suitable, although for the bridged tetrahydrophthalic anhydride for a reason that might not be equally decisive in work on every protein. Preliminary tests showed that the use of maleyl groups did not preclude our obtaining the particular coupled products that we required in a satisfactory state after deprotection. Although we would not in general recommend its use if only because it is so slowly removed, it was acceptable for our needs and had the considerable advantage that the techniques of peptide separation worked out for the digest of the enzyme modified by the other two reagents could be transferred without further development to a digest of maleylated material. In our more recent work on other proteins we have used the acetimidyl group of Hunter & Ludwig (1962) (Webster & Offord, 1972; D. E. Harris, C. J. A. Wallace & R. E. Offord, unpublished work) and note that others engaged in semisynthesis have made a similar choice (e.g. Garner & Gurd, 1975). Where solubility of the fragments is less of a problem we have used the trifluoroacetyl group of Weygand & Csendes (1952) (Borras & Offord, 1970; D. E. Harris & R. E. Offord, unpublished work) or even the Boc- group, applied to the side chains in the way described above for the α -amino groups.

In general, we favour the scheme discussed above in which there is the minimum degree of protection of the peptides. If possible the protection should, like the acetimidinyl group, interfere as little as possible with the non-covalent bonding properties of the side chains. There are signs (Corradin & Harbury, 1974; Dyckes *et al.*, 1974; C. J. A. Wallace & R. E. Offord, unpublished work) that if this condition of minimal interference is obeyed, the coupling of large peptides, far from being much slower than that of small ones,

can on occasion actually be faster. Since these couplings were in aqueous media, this effect could be the result of the bringing together of the α -carboxyl and α -amino groups by the normal interplay of non-covalent forces that stabilize the tertiary structure of the intact protein.

We believe that the approach reported in this paper, with the possible exception noted above, is applicable to the preparation of fragments for semisynthetic work in general.

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