APPENDIX

Diazoalkanes in Peptide Semisynthesis

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The accompanying paper, and the publications cited in it, indicate the need for methods of reversible protection of the carboxylic acid groups of polypeptides. Most of the methods used in conventional peptide synthesis were designed for the protection of amino acids and are unsuitable for peptides because the conditions used often threaten the stability of the peptide bond [see, for example, Backer & Offord (1968)]. Also the fact that some of the methods most commonly used do not lead to quantitative esterification is less easy to tolerate when one is protecting a polypeptide. Polypeptides often have more carboxyl groups per molecule than do the amino acids, and as a consequence the product of partial reaction will be more heterogeneous. It will then be correspondingly more difficult to separate the fully protected material from that which is partly protected. Also, it is usually less acceptable to have to discard peptide material than it is to discard amino acids.

Diazoalkanes offer one approach to a satisfactory solution. Phenyldiazomethane (Backer & Offord, 1968; Offord, 1969) and 1-diazo-2,2-dimethylpropane (Hayward & Offord, 1971) have been used successfully. We report the results of additional experiments with these two reagents and a comparison of their use in semisynthesis with *p*-methoxyphenyldiazomethane, diphenyldiazomethane and diazomethane. We also report preliminary experiments directed towards the protection of the carboxyl group without the loss of a negative charge by the use of 4-methoxy-3-sulphophenyldiazomethane.

Experimental and Results

Materials

t-Butyl cyanide (puriss) and trifluoroacetic acid (puriss) were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. 4-Methoxy-3-sulphobenzaldehyde was obtained from Kodak

Chemicals, Kirby, Lancs., U.K. All other materials were of analytical grade and came from BDH, Poole, Dorset, U.K.

Methods

Preparation of diazoalkanes. The diazoalkanes and their precursors were handled with caution in view of their possible carcinogenic activity. Diazomethane is known to be explosive; other diazoalkanes are said to be less so in general.

Where published methods have been modified in ways that are not crucial, details will be found in Supplementary Publication SUP 50071.

Diazomethane. The method of de Boer & Backer (1963) was used to prepare an approximately 0.2 M ethereal solution of diazomethane.

1-Diazo-2,2-dimethylpropane. *t*-Butyl cyanide was converted into 1-amino-2,2-dimethylpropane, thence to the urethan, and thence to the *N*-nitrosourethan by the methods described by Gerber (1952). The nitroso compound is formed as an ethereal solution, heavily contaminated with the acids used in the nitrosylation. It was not isolated as such, but extracted with five lots of an equal volume of water and then with equal volumes of 1 M-KH₂PO₄/1 M-NaOH, pH 7, for as many times as were needed to neutralize the acid. The aqueous layers were extracted in turn with another smaller sample of ether. The combined ethereal layers were kept over solid dry NaHCO₃ for 1 h, and then over Na₂SO₄ overnight. The solution was not characterized because of the possible danger of handling.

1-Diazo-2,2-dimethylpropane. The nitroso compound was converted into the diazoalkane by a minor modification of the method of Gerber (1952). In our hands these modifications had the effect of improving the yield of product.

A vacuum-distillation apparatus was set up with provision for the system to be purged by N₂ via an anti-bumping bleed of somewhat greater than normal diameter (approx. 0.2 mm). The flow of N₂ was controlled both for bleeding and for anti-bumping by a screw clip on the rubber feeder tube. Two cold-finger tubes in series, cooled by solid CO₂/propan-2-ol, were used to collect distillate. Provision was made for warming the distillation flask to 30°C, for

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magnetic stirring of its contents and for the addition of liquid reagents while the system was under vacuum. The ethereal solution of nitroso compound (from 7.5g of urethan) was introduced into the distillation flask together with a heavy magnetic stirring bar, and the ether removed under water-pump vacuum with air bleed. The system was then brought to atmospheric pressure; 20ml of ether was added and the distillation was repeated. This cycle was repeated until the distillate (which was removed from the collector traps each time) no longer lowered the pH of distilled water to an extent detectable on narrow-range pH paper. Then 10ml of fresh ether was placed in each trap, and the system connected to an oil pump capable of an ultimate vacuum of 13 Pa (0.1 Torr) or less and connected to a supply of O₂-free N₂ via the bleed. The pump was protected from the system by at least one additional cold trap. The system was purged with N₂ and, with vigorous stirring, 12ml of a solution of 1.25g of sodium in 25ml of ethanediol was added for every 7.5g of urethane that had been nitrosylated. The reaction mixture went bright orange. Vigorous stirring of the viscous mixture was continued throughout. The system was then taken to the minimum possible pressure. To achieve the minimum pressure, it was important to restrict the N₂ supply to the bleed to the absolute minimum and to lubricate any suspect ground-glass joints with ethanediol. With these precautions the diazoalkane distilled over within a few minutes, and was seen to form a strongly orange solution in the first trap and a weakly orange solution in the second trap. The mixture in the reaction vessel (which could be warmed to 30°C to assist the distillation without apparent harm) had lost nearly all its colour by the end of the distillation.

Solutions of diazoalkane could be prepared in this way up to a concentration of approx. 0.2M with a yield (based on urethan) of about 2%. They were stored in a number of small vials. If the last traces of acid were removed as described above, the solutions were stable for a few weeks at -20°C.

C. F. Hayward (unpublished work; see Supplementary Publication SUP 50071) prepared 2,2-dimethylpropan-*N*-nitroso-1-toluenesulphonamide [m.p. (decomp.) 55–56°C, with the expected N–NO stretching absorption at 1520–1540cm⁻¹]. This compound ought to be a less hazardous precursor of the diazoalkane, but repeated attempts with the method above failed to give any product whatsoever. However, the method with the nitrosourethan is so dependent on the quality of the vacuum within the apparatus (as opposed to at the point of measurement, near the intake of the pump) that it is possible that some minor change in the procedure might lead to success with the nitrosotoluenesulphonamide.

Phenyldiazomethane. Benzil was converted into the

monohydrazone, thence to azibenzil by the methods of Nenitzescu & Solomonica (1943) and Sarin & Fasman (1964). The recrystallized azibenzil was oxidized to the diazoalkane by the method of Sarin & Fasman (1969) except that the reaction mixture was stirred in the dark for 11h at room temperature. The diazoalkane was obtained as an approx. 0.2M ethereal solution, which was extracted with an equal volume of water for as many times as was necessary to give a neutral aqueous layer. The ethereal layer was dried over Na₂SO₄ and stored at -20°C in a number of small vials. It was stable for some weeks.

Diphenyldiazomethane. Solid diphenyldiazomethane was prepared by the method of Miller (1959). It was stable if stored at +2°C.

p-Methoxyphenyldiazomethane. *p*-Methoxybenzaldehyde was converted into anisalazine, by the method of Curtius (1912) and thence to the hydrazone and on to the diazoalkane by the method of Overberger *et al.* (1965). The solution, which is approx. 0.2M, was filtered through two layers of Whatman 54 paper and stored at -20°C in a number of small vials. It decomposes under these conditions with a half-time of 2–4 weeks.

Preparation of putative 4-methoxy-3-sulphophenyl-diazomethane. This method was developed by adapting a general low-temperature method for diazoalkanes proposed by Holton (1971). Attempts to prepare the reagent by the method used for the *p*-methoxy compound were unsuccessful. Although the reactions are simple, even those products that were sufficiently stable would not crystallize. We therefore rely for the characterization of our product on its spectroscopic properties, on its decolorization by acid with evolution of approximately the expected value of what appears to be N₂ and on the properties of the product formed by allowing it to react with benzoylarginine (see below). These data are sufficient to tell us that our product has the characteristics that we require for it to be useful in semisynthesis, but do not permit us to do more than draw inferences, albeit rather obvious ones, as to its chemical structure. For brevity we have omitted the word 'putative' from those points in the remainder of the text in which it should appear.

4-Methoxy-3-sulphobenzaldehyde (2.38g) was crushed and placed in a beaker. To this was added 1.5ml of anhydrous hydrazine followed by 15ml of ethanol with very vigorous stirring. The liquid immediately took on an opaque white appearance. Stirring was continued until the initially viscous mixture was flowing freely. The mixture was then left, and the white precipitate (the sodium salt of the hydrazone) filtered off and stored at -20°C. 4-Methoxy-3-sulphobenzaldehyde hydrazone (sodium salt; 0.255g) was dissolved in 12.4ml of dimethylformamide, and 3.4ml of tetramethylguanidine added to the solution. The mixture was

cooled in a bath of propan-2-ol/solid CO_2 and stirred magnetically. Lead tetra-acetate (0.63 g; 0.0013 equiv.), previously dried *in vacuo*, was added and the stirring continued for 10 min. Aq. 5% (w/v) KOH (3 ml) was then added, and after stirring for 10 min the whole mixture was filtered through Celite in a Buchner funnel (which has been precooled by standing at -20°C). The filtrate was collected in a precooled Buchner flask. The clear pink solution was stored at the temperature of propan-2-ol/solid CO_2 . It had completely decomposed after 36 h.

The lead tetra-acetate was assayed by dissolving 0.1 g of the material as obtained in 15 ml of acetic acid. To this were added 0.5 g of KI and 13 g of sodium acetate in 65 ml of water. The iodine produced was titrated against 0.01 M-sodium thiosulphate (2 equiv. of thiosulphate are required per equivalent of lead tetra-acetate.)

Assay of diazoalkanes. The ether-soluble diazoalkanes (except for diphenyldiazomethane, which was a crystalline compound and could be weighed) were assayed by allowing a measured sample to react with an ethereal solution containing an appropriate excess of benzoic acid or 3,5-dinitrobenzoic acid. The amount of acid esterified was determined either by isolating and weighing the ester or by back-titration of unconsumed acid. The water-soluble diazoalkane was roughly assayed by manometric estimation (after absorption of CO_2) of the volume of gas given off on acidification.

Approximate molar extinction coefficients were determined by drawing spectra of parallel samples to those assayed as above. The values for the coefficients were approx. 2 for the aliphatic diazoalkanes, approx. 24 for phenyldiazomethane and about 40 for the *p*-methoxyphenyl and water-soluble diazoalkanes. These values were found to be sufficient for rough working estimates of the concentration of reagent.

Preparation of substrates for protection

Peptides of lysozyme were prepared as described in the main paper. Amino-protected porcine insulin was prepared by dissolving insulin (30 mg) in 3 ml of dimethyl sulphoxide (redistilled), contained in a round-bottomed flask, and adding sufficient triethylamine (redistilled) to bring the externally indicated pH (see the main paper) to between 8 and 9. Dimethylformamide can be used as the solvent if the insulin is free of Zn. *t*-Butoxycarbonylazide (0.9 ml) was added and, if necessary, sufficient triethylamine to restore the indicated pH to its previous value. The mixture was left at room temperature overnight. It was then taken to dryness on the freeze-drier. (It is desirable, when subsequently defrosting the refrigerator chamber, to run the effluent into a dilute solution of triethanolamine to decompose the excess of azide.) The product migrated as a

triacyl-insulin on isoelectric focusing (Borras & Offord, 1970), suggesting that any Boc-imidazolyl groups that might be formed are very labile. The indicated pH did not wander during the reaction, suggesting that when samples larger than those described in the main paper are treated, triethylamine is perfectly able to control the pH. The des-(B23-B30) derivative of the amino-protected insulin was made by incubating 50 mg of the product of the previous reaction with 2 mg of trypsin under the following conditions.

A buffer was prepared by dissolving 0.45 g of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ in 20 ml of water and adding 1 ml of *N*-ethylmorpholine. HCl (0.5 M) was then added to give a final pH of 8.6. This mixture was then diluted to 50 ml with water. The protein was dissolved in 5 ml of dimethylformamide, and 5 ml of the buffer was added with stirring and cooling. The trypsin was then dissolved in the mixture, and the digestion allowed to proceed at room temperature overnight. The digest was then dialysed against frequent changes of 2 litres of distilled water at 4°C in Visking 18/32 tubing that had been exposed to boiling NaHCO_3 (1%, w/v) for 30 min. The non-diffusible residue was freeze-dried and triturated twice with 3 ml of water. The soluble fraction, which contained octapeptide, was freeze-dried, and the insoluble material was dissolved in 2 ml of dimethylformamide and subjected to gel filtration on a column (120 cm $\times$ 1.8 cm) of Sephadex LH-20 in dimethylformamide/water (17:3, v/v). The material from two peaks was collected. The first to emerge was shown by isoelectric focusing and amino acid analysis to be the desoctapeptide derivative; the other was a further quantity of octapeptide.

Esterification. Esterification in non-aqueous solvents. The substrate was converted into the carboxylic acid, as opposed to the carboxylate, form by being dissolved or suspended in cold water, which was then adjusted to pH 2.5 with 0.01 M-HCl. The liquid was freeze-dried at once. Numerous experiments with small substrates (up to six amino acid residues in length) showed that, in general, a threefold excess of diazoalkane was sufficient to bring about complete esterification in a few minutes at 0°C . Diphenyldiazomethane was exceptional in that it was extremely slow to react. Even a large excess often failed to completely esterify quite small substrates after 24 h.

Esterification in semi-aqueous solvents. Peptides (which need not have been given the acid treatment described above) were dissolved (up to 0.1 M) in either water or (if insoluble in water by virtue of other protecting groups) in the organic component of the proposed mixture. We have used methanol, ethanol or dimethylformamide, as the solubility of the starting material demands. We do not recommend dimethyl sulphoxide (although it is a very good solvent), because its use appears to lead to a heterogeneous

product, notably in peptides that have aromatic residues. The aqueous and organic components were then mixed, with cooling if the process proved to be exothermic. We normally used a mixture that contained 15% water by vol. because more of the type of substrates that we use appeared to be soluble at this sort of ratio than any other. At this ratio there was not so much water as to bring about the immediate formation of a second layer on adding the ethereal solution of diazoalkane, but there was enough to permit pH control with a glass electrode. We have on occasion used mixtures in which the quantity of water ranged from 10 to 50% by vol.

The peptide solution was then transferred to an autotitrator vessel which was surrounded by ice, and the pH brought to the desired value with aq. HCl (usually between 0.01 and 0.1 M). We found that a pH of 4.5 (standardized at 0°C) was suitable. The rate of decomposition of most diazoalkanes was prohibitively high below pH 4 and we wished to keep the pH below 5 to minimize the possibility of reactions occurring with other functional groups beside the carboxyl. The solution was stirred vigorously at all times.

The ethereal solution of diazoalkane (which was kept near the autotitrator in a receptacle cooled in propan-2-ol/solid CO₂) was then added drop by drop. The pH tended to rise after each addition, and matters were arranged so that the autotitrator was able to prevent any excursion above pH 5. At first the tendency to rise was very sharp, and the rate of evolution of N₂ and the loss of colour of the reagent was very rapid. As the addition progressed these changes slowed, and the acid uptake became less extensive, settling down to values that presumably reflected the hydrolysis of the reagent rather than its reaction with carboxyl groups. Esterification (as judged by subsequent electrophoresis of the product) was usually complete when a 30–70-fold excess of reagent over carboxyl groups had been added. Trial experiments have occasionally shown values outside that range to be necessary.

If the peptide was insoluble in the solvent system it usually went into solution during the reaction. If a great deal of ethereal solution had to be added the solution separated into two phases. If this occurred some more of the original solvent mixture was added to restore the system to a single phase.

As in the non-aqueous conditions diphenyldiazomethane was the slowest to react. It was also much slower to decompose. At pH 4 (glass electrode, 0°C) the apparent first-order rate constant of the acid uptake that accompanied its decomposition in water/dimethylformamide (3:17, v/v) in the absence of substrate was $4.18 \times 10^{-4} \text{ s}^{-1}$. The corresponding value at pH 3 was $1.75 \times 10^{-3} \text{ s}^{-1}$. By contrast the decomposition of the aliphatic diazoalkanes was practically instantaneous at pH 3, and still too fast for the autotitrator to follow at pH 4.

After the reaction was judged to be complete, the mixture was taken briefly to pH 3.5 to decompose any traces of diazoalkane and then to pH 7 (with 0.1 M-pyridine in water, or any other convenient base). The ether was removed on the rotary evaporator. A copious precipitate, which we believe to be of by-products derived from the diazoalkane, often formed during this operation. This precipitate was removed by centrifugation, and after testing for peptide material, discarded. The remaining solution could then be taken to dryness and the peptide material extracted from most of the remaining by-products into pyridine/water (1:1, v/v). An alternative, which was particularly necessary if the peptide contained tryptophan (see below), was to free the solution completely from by-products by direct application of the supernatant from the centrifugation to a column of Sephadex LH-20 in dimethylformamide, ethanol or whatever aqueous mixture was dictated by the solubility of the peptide materials.

Esterification with the water-soluble diazoalkane. The reagent is prepared in a very basic aqueous solution. It decomposes rapidly on handling at room temperature and for these preliminary experiments it was used without purification. The solution was added dropwise to a vigorously stirred solution of benzoyl-arginine (54 mM in ethanol/water, 17:3, v/v) which was kept between pH 5 and 5.5 with 0.5 M-HCl in an autotitrator cell at 0°C. After a tenfold excess of reagent had been added (based on the hydrazone), the mixture was dried-down and subjected to electrophoresis at pH 3.5 [28 mg (including salts) in 10 μ l/cm of Whatman 3MM paper] to remove unchanged benzoylarginine. The ester was detected by staining a guide-strip for arginine-positive material. It moved as a result of endosmosis only, and the benzoylarginine travelled toward the negative electrode. A test spot of the ethyl ester moved even more rapidly to the negative. The ester was eluted from the paper with pyridine/water (1:9, v/v).

Assessment of the percentage of t-amyl ester formed with 1-diazo-2,2-dimethylpropane

Hayward & Offord (1971) pointed out that the formation of the t-amyl ester by the primary diazoalkane occurs as the result of the rearrangement of the carbonium ion first formed on the protonation of the reagent. They suggested that the rearrangement would be favoured in media of high dielectric constant. The semi-aqueous conditions are thus to be preferred. The diazoalkane is added in ethereal solution, and we sought to determine whether the progressive decrease in dielectric constant had a serious effect on the reagent. (If it did, it would be simple enough to exchange the solvent in which the reagent is stored.) We therefore esterified 1 μ mol of samples of Boc-leucine and glutamic acid in

solvents that initially contained 15% water by vol. We treated the totally esterified products with anhydrous trifluoroacetic acid for 40 min at 20°C and then subjected the material to paper electrophoresis at pH 6.5; the papers were then stained with Cd/ninhydrin. We used the rough working assumptions that all the tertiary ester would be deprotected, and that the colour yield of the remaining primary ester is approximately that of the free amino acid. After heating the papers to 90–100°C for 15 min, there was very little difference in shade between the two spots. The colours were eluted by cutting out the spots and soaking in ethanol. Intensities were determined at 500 nm. We found the extent of the rearrangement, assessed in this way, to vary between 94 and 100%. Even the 94% value is not prohibitively low; a molecule with six carboxyl groups would still give a 70% yield of totally deprotected product.

Assessment of damage on protection and deprotection. Non-aqueous conditions. Side reactions, which seemed most likely to be due to *N*-alkylation, were a serious complication when unprotected peptides were treated under non-aqueous conditions. Peptide M7 (see Table 2 of the main paper) when treated with diazomethane as described above gave, in addition to the expected ester, three other forms, electrophoretically distinguishable at pH 8.9, which stained poorly or not at all with ninhydrin. Peptide M3, similarly, gave a high yield of the side product that Hayward & Offord (1971) concluded was due to alkylation of the imidazolyl function. Peptide M6 lost the power to give the Ehrlich reaction for tryptophan when treated in this way with phenyldiazomethane.

Semi-aqueous conditions. Offord (1969) and Hayward & Offord (1971) have shown that the side reactions are much less serious in semi-aqueous media under pH control. Even unprotected peptides will normally revert to the starting material after protection and deprotection. The principal exception noted by these authors was the histidine-containing peptide M3, which gave small but visible quantities of the apparently alkylated product (much less than was so in the non-aqueous conditions) when treated with 50-fold excesses of diazoalkane and then deprotected. By contrast with the non-aqueous conditions, there was no visible loss of tryptophan when using the semi-aqueous method. Good recoveries of tryptophan could be obtained on acid deprotection (see the main paper) provided that the last traces of the by-products of esterification were removed before the addition of acid. If this precaution was neglected, amino acid analysis after deprotection plus basic hydrolysis showed losses of tryptophan ranging from 36% (a trace of by-product) to 100% (heavy contamination).

The application of an appropriate protecting group to the side chain of histidine (see the main paper)

abolished its tendency to give the side reaction, and [*N*^ε-Boc-*N*^α-Boc-NH-CH(CF₃)-histidine]peptide M3 reverted to material that was electrophoretically indistinguishable from peptide M3 after esterification with *p*-methoxyphenyldiazomethane or phenyldiazomethane and subsequent acid deprotection. Although the protection of the amino groups does not seem to be essential with the semi-aqueous conditions, it seems prudent, and quite easy, to do so. We normally use the range of groups mentioned in the main paper, except that we favour the acetimidyl group in place of the maleyl group.

We find, in addition to damage of the sort mentioned above, additional problems when protecting and deprotecting insulin. Of all the diazoalkanes tried only *p*-methoxyphenyldiazomethane will give a readily crystallizable product on protection and deprotection. The quality of the crystalline material is greatly improved if the molecule is first protected against *N*-alkylation, even if only the amino groups are so treated.

Selective deprotection by trypsin. Each of the diazoalkanes was used to form the ester of *N*^ε-benzoyl-arginine (except for diphenyldiazomethane when, for trivial reasons, a sample of *N*^ε-acetylarginine was used). All gave a product of the predicted (Offord, 1966) electrophoretic mobility, staining distinctly more red with the Sakaguchi stain than the starting product. Samples (50 nmol of each) were incubated in 100 μl of 0.5% NH₄HCO₃ solution at 37°C for 30 min, with 5 μl of a solution (2.5 mg/ml) of trypsin. All samples reverted to the starting product. Controls, in which trypsin was omitted, showed no deprotection except for a trace of starting product in the sample of 4-methoxy-3-sulphobenzyl ester. Thus, in spite of the steric hindrance of some of the more bulky groups, and the negative charge of the sulphonyl ester, all are readily cleaved by trypsin and so are, in this respect, suitable for semisynthesis.

The data in Table 3 of the main paper indicate the general applicability of trypsin for selective deprotection. In addition, we have tested the ability of trypsin to de-esterify the α-carboxyl group of the C-terminal arginine in Boc₂-desooctapeptide insulin after esterification with diphenylmethyl and *p*-methoxybenzyl groups. The autotitrator was used to follow the reaction. The *p*-methoxybenzyl group is removed with an initial rate of 0.14 μmol/min at pH 8, 20°C, in 10 ml of dimethylformamide/water (1:1, v/v), 0.01 M in CaCl₂, when the quantity of substrate was 0.79 μmol, and the quantity of trypsin 0.002 μmol. (By contrast, trypsin when acting as a proteinase cleaves at the arginine residue much less readily. The complete removal of the octapeptide from amino-protected *p*-methoxybenzylated insulin required 16 h at 20°C and a 20-fold increase in the concentration of trypsin.)

The diphenylmethyl ester of the desooctapeptide

insulin was only soluble in dimethylformamide/water mixtures that contained 33 % water or less, as at these concentrations trypsin was not active against the ester.

General deprotection. The deprotection conditions for the methyl, benzyl and diphenylmethyl groups are well known. The *t*-amyl ester formed by the 1-diazo-2,2-dimethylpropane behaves much like the *t*-butyl ester. We find that the acid lability of the *p*-methoxybenzyl group depends on the type of carboxyl group esterified (see the main paper for details), but that it is in general practical to remove it with anhydrous trifluoroacetic acid (c.f. Stelakatos & Argyropoulos, 1970). In one of its more acid-labile forms, the *p*-methoxy ester of benzoylarginine, it was found to resist exposure to dry HCl (1.8M) in anhydrous dimethylformamide for 30 min at 20°C. The Boc group was almost completely removed from *N*^ε-Boc-lysine methyl ester under these conditions, which offer the prospect of a useful degree of selectivity.

The 4-methoxy-3-sulphobenzyl ester of benzoylarginine was, as judged electrophoretically, completely deprotected in HBr/trifluoroacetic acid (60 min at 20°C; conditions of Stewart & Young, 1969) and about 80 % deprotected by anhydrous trifluoroacetic acid alone (16 h at 20°C). Aq. trifluoroacetic acid under the same conditions led to very little deprotection, as did 2M-acetic acid for 1 h at 0°C. The effect of the sulphonic acid group appears to have been to decrease lability to acid. Surprisingly, there was a marked increase in lability to aqueous base. The group was completely removed from benzoylarginine by aq. 0.1M-NH₃ (16 h at 20°C). There was perceptible deprotection in aq. *N*-ethylmorpholinium and ammonium buffers at pH values between 7 and 8 though none below pH 7, and little or none when treated with a 2 % solution of *N*-ethylmorpholine in dimethyl sulphoxide at 20°C for 16 h. These observations led to an investigation of the base lability of the *p*-methoxybenzyl ester of benzoylarginine. It resisted overnight treatment at 20°C under all the basic conditions mentioned above, except for aq. 0.1M-NH₃, which led to some slight degree of deprotection.

Other methods. All other methods were as described or cited in the main paper.

Discussion

In order to be suitable for general use a reagent must be reasonably convenient to prepare, store and handle. None of the diazoalkanes mentioned here is positively ruled out on these grounds. They are all quite simple to prepare except for 1-diazo-2,2-dimethylpropane, the use of which cannot at the moment be avoided if azide couplings are intended (Hayward & Offord, 1971). All can be stored for

some weeks at -20°C, except the water-soluble reagent, which promises to have compensating advantages. All diazoalkanes must be treated with respect, but in the 8 years of fairly intensive use of this class of reagent in this laboratory there has not been any noticeable mishap. We believe this to be the common experience of the many laboratories that use diazoalkanes for organic synthesis. As far as the latent effects in man of chronic low-level exposure to diazoalkanes and their precursors are concerned, only time will tell.

In order to be suitable specifically for peptide semisynthesis the reagents must meet a number of additional criteria. First, for the reasons given in the introduction, they should give quantitative protection under mild conditions. Only diphenyldiazomethane, which reacts very slowly, is in danger of failing to meet this requirement. A second criterion is that, when tryptic fragments are used, the protected material can be selectively deprotected at its α -carboxyl group. Therefore the ester group must be intrinsically capable of tryptic cleavage and also not make the peptide so apolar that it cannot be dissolved in a solvent in which trypsin will act. The experiments with the acylated arginines show that, perhaps surprisingly, all the esters are capable of being cleaved. Diphenyldiazomethane makes larger protected fragments apolar to an almost prohibitive extent. Diazomethane (if we exclude for the moment the water-soluble reagent) makes the protected fragment the least apolar. As the main paper shows, this problem does not prohibit the use of the middle range of the diazoalkanes, but larger fragments, though soluble enough in the totally protected state for tryptic esterolysis, are sufficiently insoluble to make characterization troublesome and at times uncertain. This is one reason why we have paid some attention to protection by the polar 4-methoxy-3-sulphobenzyl group. We also hope, if we protect the carboxyl group without loss of the negative charge, for some of the possible advantages cited for amino protection by positively charged groups mentioned in the penultimate paragraph of the main paper.

Thirdly, the protection and final deprotection of the group ought not to damage the polypeptides in any way. Provided that the amino group and imidazole groups are protected, and that the side products of esterification are removed before deprotection we find little evidence of any such damage. Traces of materials deriving from the column or paper separation of the peptides do not appear to interfere at any stage.

To suggest that diazoalkanes are available that meet all these criteria is not to say that there is no need for further development. We believe that there is a considerable need for other methods of esterification to be introduced into peptide semisynthesis

and for carboxyl-protecting groups, whether introduced by diazoalkanes or otherwise, that are stable to acid but labile, without side reactions, to alkali.

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