Use of Hydrogen Fluoride in Merrifield Solid-Phase Peptide Synthesis[1]

Sir:

Sakakibara and Shimonishi[2] have shown that treatment of the synthetic fully blocked peptide oxytocin with anhydrous hydrogen fluoride and excess anisole removes the N-carbobenzyloxy, S-benzyl, and S-*p*-methoxybenzyl protecting groups from the peptide. Sakakibara has also removed a wide variety of other peptide blocking groups by these conditions.[3]

We have found that procedures similar to those of Sakakibara and Shimonishi can be used to remove the completed peptide from the resin in Merrifield solid-phase peptide synthesis.[4] Also, we have found that under the same reaction conditions the N-nitro group is quantitatively removed from nitroarginine residues of the protected peptide (see also ref 5).

These experiments were carried out on a Monel vacuum line fitted with Hoke nickel diaphragm valves.[6] The reaction vessels (1.5 × 15 cm) were translucent Fluorothene tubes attached to the line by standard SAE refrigeration flare fittings. Hydrogen fluoride (Matheson) was purified on this line by distillation under vacuum (oil pump) into a liquid nitrogen cooled reaction vessel containing cobalt trifluoride as drying agent. Hydrogen fluoride was distilled under vacuum from this vessel into the liquid nitrogen cooled reaction vessel containing the protected peptide–resin. Removal of hydrogen fluoride upon termination of the reaction was effected by a similar vacuum distillation. During the reaction and during removal of the hydrogen fluoride the mixture was stirred with a Teflon-coated magnetic stirring bar.

Bradykinin was prepared to demonstrate the method. The *t*-butyloxycarbonyl (*t*-BOC) amino acids were obtained from Cyclo Chemical Corp. and were used without further purification.

The protected peptide–resin, *t*-BOC-nitro-L-Arg-L-Pro-L-Pro-Gly-L-Phe-O-benzyl-L-Ser-L-Pro-L-Phe-nitro-L-Arg-resin, was prepared using Merrifield's procedure of peptide synthesis.[4] The *t*-BOC protecting groups were removed with 1 *N* HCl in anhydrous acetic acid. The resulting hydrochlorides were neutralized with 10% triethylamine in chloroform. The peptide bonds were formed with N,N′-dicyclohexylcarbodiimide in methylene chloride (except when *t*-BOC-nitro-L-arginine was added to the growing peptide chain, in which case dimethylformamide was used as solvent) followed by an ethanol rinse. The synthesis was carried out by an automatic machine[7] similar to that described by Merrifield.[8]

The protected peptide–resin was treated with hydrogen fluoride and excess anisole at room temperature for 1 hr. Generally, a tenfold molar excess of anisole was used, based on the number of bonds to be broken, although as little as a twofold excess has been successfully used. The volume of hydrogen fluoride used varied from 3 to 10 ml, but was always at least ten times the volume of anisole.

After evaporation of the hydrogen fluoride from the reaction mixture, the bradykinin was separated from the resin by repeated washing of the resin with 1% acetic acid. Amino acid analysis of the solution showed it to contain 102 ± 5% of the arginine residues originally present as nitroarginine on the resin. No nitroarginine was detected after the hydrogen fluoride–anisole treatment.

The crude peptide was purified by countercurrent distribution in 2-butanol–trifluoroacetic acid–water (100:1:100).[9] Bradykinin constituted the major peak and had a partition coefficient of 1.4 in this system. The yield of bradykinin based on arginine in the crude mixture was 66%.

This material showed a single ninhydrin- and Sakaguchi-positive spot upon paper electrophoresis at pH 2 and 5.5. The amino acid ratios[10] (uncorrected) were: Arg 2.00, Pro 3.00, Gly 0.97, Ser 0.90, Phe 1.96 (all ± 5%). The peptide possessed full biological activity as measured by the rat uterus contraction assay[11] (103 ± 9% of the activity of authentic bradykinin). Both the sample and the authentic bradykinin with which it was compared were assayed as the tri(trifluoroacetate) salts.

Hydrogen fluoride is known to cause an N- to O-acyl shift at serine and threonine residues in proteins (during several hours)[12a] and dipeptides (during several days).[12b] Since bradykinin contains a serine residue, the four countercurrent distribution peaks comprising impurities were examined to see whether they had arisen from bradykinin by an N- to O-acyl shift. None of these peaks gave the correct amino acid analysis for bradykinin, nor did their electrophoretic mobilities at pH 2 change after mildly alkaline treatment.[12b] Thus, the N- to O-acyl shift did not occur to a significant extent under these conditions.

This procedure for removal of peptide from the resin in the Merrifield synthesis has been in routine use in our laboratory for the past year, during which time no difficulties have been encountered. It offers advantages over existing techniques in that most blocking groups are removed simultaneously with the resin.[2,3] Also, it should be emphasized that hydrogen fluoride is extremely easy and convenient to use, once a suitable vacuum line has been constructed. Some of the other peptides which have been prepared in this laboratory by Merrifield synthesis followed by hydrogen fluoride–anisole treatment are: (a) a tetrapeptide, L-pyrrolidonecarboxylic acid-L-Phe-L-Ala-L-Arg,[13] (b) a hexapeptide, Gly-L-Lys-L-Asn-L-Lys-Gly-L-Arg,[14] (c) and a pentadecapeptide, L-CySH-L-Ala-L-Val-L-CySH-*im*-benzyl-L-His-L-Ala-L-Val-L-Ala-L-Lys-L-Ala-L-Ala-L-Ala-L-Ala-L-Ala-L-Ala.[15]

(1) Supported by Grant No. HD 01262 (National Institutes of Health) and GB 2892 (National Science Foundation) to Professor Martin D. Kamen.

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(3) S. Sakakibara, personal communication, July 1965.

(4) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).

(5) J. Lenard, *J. Org. Chem.*, **32**, 250 (1967).

(6) H. H. Hyman, Ph.D. Thesis, Illinois Institute of Technology, 1960. A diagram of the line can be obtained from the Information Division, Argonne National Laboratory, Lamont, Ill., ANL negative 120-4450.

(7) A. B. Robinson and M. D. Kamen, unpublished.

(8) R. B. Merrifield, *Science*, **150**, 178 (1965).

(9) R. B. Merrifield and J. M. Stewart, personal communication.

(10) Amino acid analysis was performed by Mrs. R. M. Smith; see K. Dus, S. Lindroth, R. Pabst, and R. M. Smith, *Anal. Biochem.*, **14**, 41 (1966).

(11) We are grateful to Dr. J. M. Stewart and Professor R. B. Merrifield for performing this assay.

(12) (a) J. Lenard and G. P. Hess, *J. Biol. Chem.*, **239**, 3275 (1964); (b) S. Sakakibara, K. H. Shin, and G. P. Hess, *J. Am. Chem. Soc.*, **84**, 4921 (1962).

(13) R. F. Doolittle and A. B. Robinson.

(14) T. Flatmark and A. B. Robinson.

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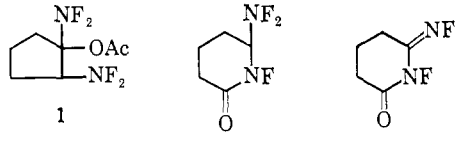
Rearrangements of Organic Fluoramines. Preparation of 3-Difluoramino-2-fluoro-2-azacyclohexanone and 3-Fluoro-3-(3-carbomethoxypropyl)diazirine

Sir:

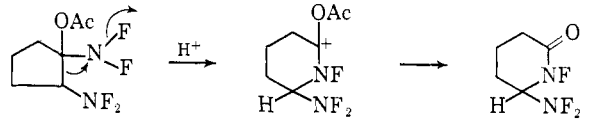
During a study of the properties of organic difluoramino compounds we prepared 1,2-bis(difluoramino)-1-acetoxycyclopentane (**1**), bp 42° (1 mm), mixture of *cis* and *trans* isomers by vpc and ^{19}F nmr (*Anal.* Found: C, 36.88; H, 4.69; N, 12.14; F, 33.4), from 1-acetoxycyclopentene and tetrafluorohydrazine.[1,2] Treatment of this bis(difluoramine) (**1**) with 96 or 100% sulfuric acid or with fluorosulfonic acid produced (60–70%) 3-difluoramino-2-fluoro-2-azacyclohexanone (**2**) as the only isolable organic product. Samples of **2** were purified by chromatography on silica gel. *Anal.* Found: C, 35.78, H, 4.46, N, 17.02, F, 33.6, infrared λ_{max} 5.75 (C=O) and 10.8–12 μ (NF). The ^{19}F nmr spectrum[3] of **2** was deceptively simple; two apparent quartets at −1428, −1444, −1460, −1476, and at +2268, +2284, +2304, +2320 cps were observed in CCl_4 solution. The proton spectrum exhibited an apparent quartet of multiplets centered at δ 5.29 ($HCNF_2$)[1] and a broad peak 100–170 cps downfield from TMS due to the trimethylene chain.[4] The timethylene chain is not attached to >NF since a shift of greater than 200 cps from TMS would be expected.[5]



1 2 3

Further insight into the spectra of **2** was gained when ^{19}F homonuclear decoupling confirmed couplings of about 25 cps between the NF and NF_2 nuclei. That the >NF should appear as a quartet due to a coincidence of ^{1}H–^{19}F and ^{19}F–^{19}F couplings was now evident; however, the NF_2 group should only be a triplet. The only tenable hypothesis was that the NF_2, being on an asymmetric carbon, had nonequivalent fluorines only slightly shifted relative to one another. The nonregular splitting of the CH quartet and ^{19}F spectra run on solutions of trifluoroacetic acid confirmed the hypothesis. The familiar AB quartet of NF_2 on asymmetric carbon[1,5] was now apparent: each of the strong central resonances was a triplet from the fortuitously equal effects of proton and >NF couplings. Homo- and heteronuclear decoupling experiments on trifluoroacetic acid solutions confirmed all the assumptions made in the spectral interpretations of this deceptive ABMX system.

Formation of **2** from **1** can be rationalized as a rearrangement occurring with acid-catalyzed loss of fluoride ion from nitrogen.[6] Other routes from **1** to **2** are conceivable, of course.

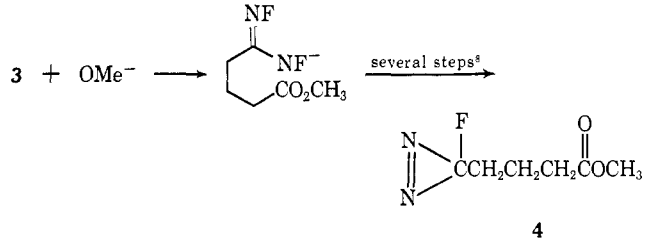


1 2

Dehydrofluorination of **2** with triethylamine in methylene chloride produced fluorimine **3** as a mixture of *syn* and *anti* isomers (*Anal.* Found: C, 40.52, H, 4.34, N, 18.11). The ^{19}F nmr spectrum of **3** consisted of peaks at ϕ −29.4 and +68.9 due, respectively, to the C=NF and the NF of the *anti* form, and of much weaker peaks at ϕ −18.9 (doublet, J_{FF} = 96 cps) and +61.9 (doublet, J_{FF} = 96 cps) due to the same functional groups in the *syn* isomer.

Treatment of either **2** or **3** with excess sodium methoxide in methanol produced 3-fluoro-3-(3-carbomethoxypropyl)diazirine (**4**); λ_{max} (cyclohexane) 358, 341 mμ (ϵ 234, 203), respectively.[7] *Anal.* Found: C, 45.10, H, 5.97, N, 17.22. The ^{19}F nmr spectrum of **4** had the C*F* peak at ϕ +139.2 (triplet, J_{HF} = 8 cps), and the infrared spectrum had an ester carbonyl absorption at 5.70 μ and the strong diazirine absorption at 6.39 μ.[7]

The mechanism for the formation of diazirine **4** undoubtedly involves cleavage of **3** to produce the intermediate shown; the steps involved in going from this intermediate to **4** are the same as formulated for the synthesis of 3-halodiazirines from amidines and sodium hypochlorite.[8]



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(1) R. C. Petry and J. P. Freeman, *J. Am. Chem. Soc.*, **83**, 3912 (1961); A. J. Dijkstra, J. A. Kerr, and A. F. Trotman-Dickenson, *J. Chem. Soc., Sect. A*, 582 (1966).
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(3) At 40 Mc with CCl_3F as internal standard; ϕ values are given in parts per million from internal CCl_3F.
(4) At 60 Mc, Varian A-60 spectrometer.
(5) See F. A. Johnson, C. Haney, and T. E. Stevens, *J. Org. Chem.*, in press, for a detailed discussion of the nmr spectra of 1,2-bis(difluoramino)-1,2-diphenylethane.
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(8) W. H. Graham, *J. Am. Chem. Soc.*, **87**, 4396 (1965).