^{19}F Nuclear Magnetic Resonance for the Control of Peptide Synthesis

E. Bayer,* P. Hunziker, M. Mutter, R. E. Sievers[1] and R. Uhmann

Contribution from Chemisches Institut der Universität, Tübingen, Germany, and Aerospace Research Laboratories, ARL/LJ, Wright-Patterson Air Force Base, Ohio 45433. *Received September 14, 1970*

Abstract: The use of ^{19}F nuclear magnetic resonance for the determination of error sequences occurring during a solid-phase peptide synthesis is described. The method allows fast determination of the yield in the individual coupling steps and analysis of the final product mixture.

The synthesis of a homogeneous peptide by the solid-phase method is impossible because complete coupling reactions are difficult to obtain. Both failure and truncated sequences may arise as a result of the coupling reaction being incomplete, and the separation of these compounds with incorrect sequences may be difficult.[2] Direct and fast methods for the identification and quantitative measurement of the truncated sequences formed have to be developed in order to follow and control the course and success of a peptide synthesis.

The use of ^{19}F nmr spectroscopy is proposed here as a solution to this problem. It has been shown by Sievers, *et al.*,[3] that fluorine resonance may be used for the identification of amino acids. The trifluoroacetyl group (TFA) was demonstrated to be a suitable sensor for this purpose. The main advantage of employing fluorine resonance over proton resonance is the approximately tenfold larger range of the chemical shift displayed by the fluorine nuclei.

In the case of the trifluoroacetyl compounds, one singlet is obtained for each trifluoroacetyl (TFA) group. This allows easy interpretation of the spectra, especially when compared with the proton spectra which are very complicated even for some simple amino acids due to spin splitting. It has already been shown[3] that the signals of trifluoroacetyl groups of different amino acids appear at characteristic field positions.

Results and Discussion

A knowledge of the chemical shift of the various amino acids is a prerequisite for the interpretation of the spectra obtained from peptides. In Table I, the chemical shifts of some trifluoroacetylated amino acids are given. Figures 1 and 2 demonstrate that even closely related amino acids like aspartic acid and asparagine or tyrosine and phenylalanine can be clearly distinguished by their chemical shifts.

The solid-phase synthesis of the peptide H-Ile-Ala-Val-Gly-OH was quantitatively monitored by ^{19}F nmr spectroscopy. After each coupling step, a sample of the resin was taken, the product was cleaved from the support and trifluoroacetylated, and ^{19}F nmr spectra (Figures 3–6) were recorded. By integration of the peak area, the yields of the various peptides were calculated. Figure 3 shows that the coupling to give the dipeptide H-Val-Gly-OH (II) has proceeded to 90% completion. The coupling of alanine to valine reached approximately 70%; no significant coupling of valine to the residual glycine occurred. Figure 4 shows that

Table I.[a]

^{19}F chemical shifts of amino acids, ppm, relative to CF_3COOCH_3	
N-TFA-Gly-OH	0.61
N-TFA-Ala-OH	0.49
N-TFA-Val-OH	0.08[b]
N-TFA-Leu-OH	0.31
N-TFA-Ile-OH	0.10
N-TFA-Asp-OH	0.56
N-TFA-Glu-OH	0.47
N-TFA-Asn-OH	0.72
N-TFA-Gln-OH	0.76
N-TFA-Phe-OH	0.49
N-TFA-Tyr-OH	0.46
N-TFA-Try-OH	0.44
N-TFA-Ser(OBzl)-OH	0.29
N-TFA-Thr(OBz)-OH	0.22
N-TFA-Cys(SBzl)-OH	0.33
N-TFA-cystine	0.36
N-TFA-Met-OH	0.37
N-TFA-Arg-OH	0.58

[a] Solvent, acetone, internal standard, methyl trifluoroacetate. The instrument used was the Varian HA-60 operated at 56.4 MHz. [b] The value shown here is correct; the value published earlier in ref 3 was a typographical error.

65% tripeptide III, 25% dipeptide II, and 10% glycine I were present. The final coupling of isoleucine occurred in approximately 85% yield. The dipeptide did not react further, so in this synthesis obviously the truncated sequences are dominant. However, it seems as if the glycine content decreased to 5% suggesting that a failure dipeptide Ile-Gly was formed. This could not be distinguished from the tetrapeptide. The yields measured by the ^{19}F nmr method agree with the results obtained by separation of the product obtained after coupling of Ile by ion-exchange chromatography (Table II).

A comparison of the chemical shifts of the peptides in Figures 3–6 with the corresponding N-terminal N-TFA amino acids in Table I demonstrates that in these cases the chemical shifts differ by less than 0.05 ppm. By ^{19}F nmr it is thus possible not only to achieve a quantitative analysis of a peptide mixture obtained during a synthesis, but also to identify the various com-

(1) Work partly done while R. E. S. was on leave from the Aerospace Research Laboratories.

(2) E. Bayer, H. Eckstein, K. Hägele, W. A. König, W. Brüning, H. Hagenmaier, and W. Parr, *J. Amer. Chem. Soc.*, 92, 1735 (1970).

(3) R. E. Sievers, E. Bayer, and P. Hunziker, *Nature (London)*, 223, 179 (1969).

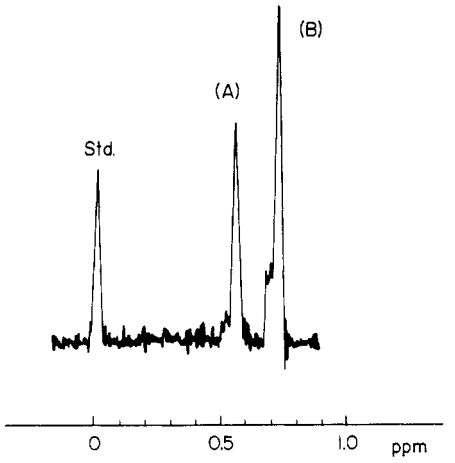

Figure 1. ^{19}F nuclear magnetic resonance spectrum of a mixture of N-TFA-Asp-OH(A) and N-TFA-Asn-OH (B), dissolved in acetone. Instrument, Varian HA-60 spectrometer, operated at 56.4 MHz. Internal standard, methyl trifluoracetate.

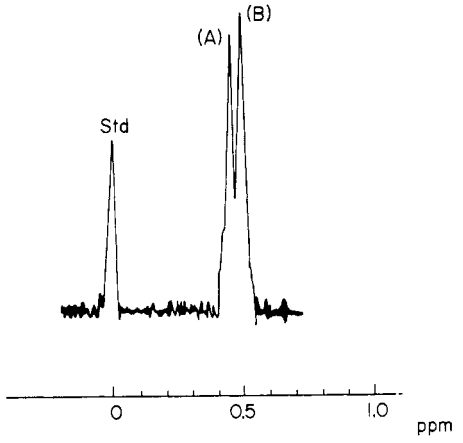

Figure 2. ^{19}F nuclear magnetic resonance spectra of mixture of N-TFA-Tyr-OH (A) and N-TFA-Phe-OH (B), dissolved in acetone.

ponents according to their N-terminal amino acid. Therefore, in the case of simple peptide mixtures a tedious separation is not necessary in order to identify the components. In contrast with the amino acid analysis for example, the ^{19}F nmr is a differential method; the truncated sequences and, in favorable cases, the failure sequences are also directly measured. Because

Table II. Ion-Exchange Chromatography of the Peptide Mixture after the Coupling of Ile.[a]

	I	II	III	IV
Glycine		1.0	1.0	1.0
Valine		1.0	1.0	0.99
Alanine			0.9	1.0
Isoleucine				0.98
Yield in % of I–IV as measured by ion-exchange chromatography	3–7	16–24	11–15	53–62
Yield of I–IV by nmr, %	6	24	14	56

[a] The peptide mixture was cleaved from the solid support and a sample subjected to ion-exchange chromatography on Dowex 50-X4 (2 × 80 cm column). The peptides II–IV obtained were characterized by amino acid analysis. The dipeptide Val-Gly was not completely separated from the tripeptide Ala-Val-Gly.

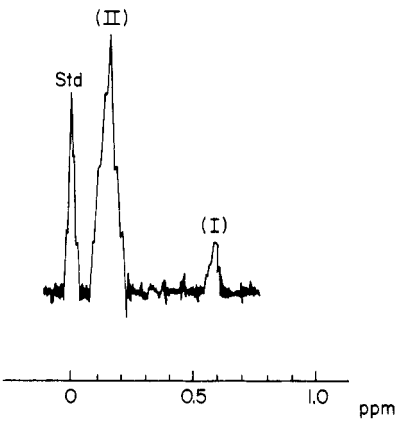

Figure 3. ^{19}F nuclear magnetic resonance spectra of the TFA derivatives of the reaction products after the first coupling step to form Val-Gly (II).

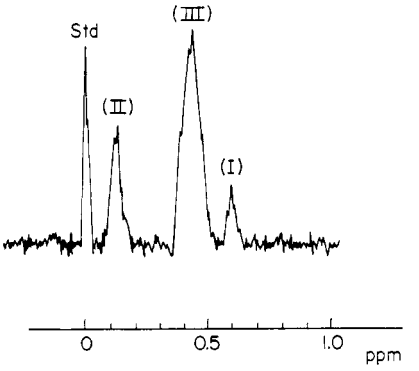

Figure 4. ^{19}F nuclear magnetic resonance spectra after the second coupling step to form Ala-Val-Gly (III).

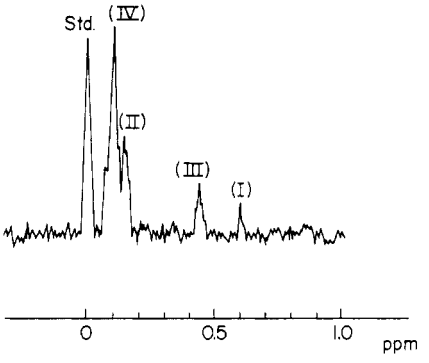

Figure 5. ^{19}F nuclear magnetic resonance spectra after the third coupling step to form Ile-Ala-Val-Gly (IV).

the position of the ^{19}F nmr signal is primarily dependent upon the N-terminal amino acid, and only to a lesser extent upon the remainder of the molecule, the method is applicable to larger peptides.

The penultimate amino acid influences to a slight degree the position of the ^{19}F nmr signals. The signals of all amino acids are shifted to slightly lower field position, but retain the same differences with respect to one another. This fact may be illustrated in a further example. The peptide BOC-Val-Thr(Bzl)-Val-Leu-Thr-(Bzl)-Ala-Leu-Gly-OCH_3 (V), a partial sequence of myoglobin, was synthesized by the solid-phase method. Cleavage of the peptide from the solid support was car-

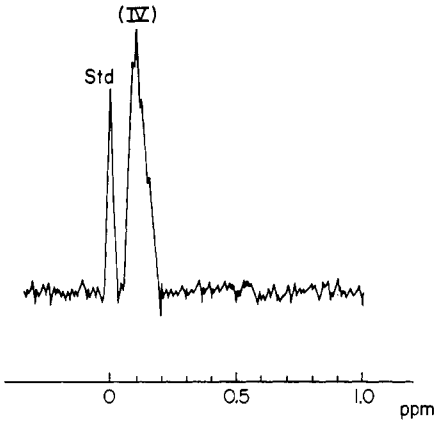

Figure 6. ^{19}F nuclear magnetic resonance spectra of the purified tetrapeptide (IV).

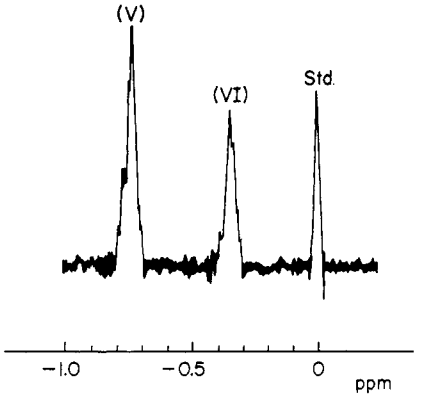

Figure 7. ^{19}F nuclear magnetic resonance spectra of a mixture of the TFA-octapeptide (V) and a TFA-heptapeptide (VI) in the ratio 65:35, dissolved in hexamethylphosphoric acid triamide.

ried out by transesterification. Partial purification of the crude product was made by gel filtration. The material obtained was found by thin-layer chromatography to be a mixture of at least two compounds, which could not be readily separated in quantities. However identification was achieved by mass spectrometric analysis of the peptide mixture after acetylation and permethylation. End group determinations *via* the dansyl derivatives were in agreement with the results by ^{19}F nmr. A sample was deprotected and then trifluoroacetylated, and the following results were obtained (Figure 7): peptide V was accompanied by 35% of the truncated sequence BOC-Thr(Bzl)-Val-Leu-Thr(Bzl)-Ala-Leu Gly-OCH_3 (VI). Apparently the elimination of the protective group preceding the last coupling step was not complete.

Despite the high degree of similarity between the peptides V and VI, a difference in the chemical shift of 0.38 ppm, the same as that between the N-terminal amino acids (TFA-Val-OH and TFA-Thr(Bzl)-OH) in the same solvent, was observed in the ^{19}F nmr spectrum.

The ease of preparation of the required derivatives, the speed with which the spectra can be obtained, and the simplicity of interpretation recommend the routine use of ^{19}F nmr for the determination of the homogeneity of the products of peptide synthesis and the yield in individual coupling steps. Of course, this new method can also be used for the control of any other type of peptide synthesis, such as fragment condensation, where the problem of incorrect sequences is less complicated than in the case of solid-phase synthesis.

Experimental Section

Trifluoroacetylation with Trifluoroacetic Anhydride[4] (TFA-anhydride). Ten milligrams of an amino acid or of a peptide was dissolved in 3 ml of trifluoroacetic acid. The solution was then cooled to −20° and 3 ml of trifluoroacetic anhydride was added with stirring. The mixture was allowed to warm up to room temperature and, after 1 hr, was evaporated to dryness.

Trifluoroacetylation with Methyl Trifluoroacetate[2] (Methyl-TFA). The peptide esters were dissolved in 5 ml of methanol and adjusted to pH 7.5–8 by addition of triethylamine. Three milliliters of methyl trifluoroacetate was added with stirring, and the solution was left at room temperature for 12 hr. After evaporation to dryness *in vacuo*, the residue was extracted with a mixture of equal amounts of ethyl acetate and water. The organic phase was evaporated *in vacuo*.

(4) F. Weygand and R. Geiger, *Chem. Ber.*, **89**, 1543 (1956).

Synthesis of H-Ile-Ala-Val-Gly-OH. *tert*-Butyloxycarbonylglycine (1.6 mmol) was esterified to 5 g of chloromethylated Bio-Beads SX-2, 200–400 mesh (Bio-Rad Laboratories). After deprotection, the amount of esterified glycine was determined by titration of the free amino groups and found to be 0.26 mmol/g. The further steps were carried out as previously described.[6] After each coupling step, 1 g of resin was treated with hydrogen bromide in trifluoroacetic acid. The crude product obtained after filtration and evaporation was trifluoroacetylated with TFA-anhydride. The ^{19}F nmr spectra were measured and the yield of each coupling determined from the peak areas. The crude products were subjected to ion-exchange chromatography on a 2 × 80 cm column of Dowex 50-X4 (200–400 mesh) using 0.1 *M* pyridine acetate buffer of pH 4. The flow rate was 1 ml/min and 3-ml fractions were collected. Aliquots of 0.2 ml were analyzed by the ninhydrin method.[6] Fractions containing the same substance were pooled and their contents identified by paper chromatography and amino acid analysis after acid hydrolysis.[6] The amino acid composition of the tetrapeptide was: Gly, 1.0; Val, 0.99; Ala, 0.99; Ile, 1.0. The purified peptides were trifluoroacetylated; with these pure trifluoroacetylated products the peaks in the ^{19}F nmr spectra of the crude products were correlated to the various peptides, as shown in Figure 5.

Synthesis of the Octapeptide. The synthesis was carried out according to the method described by E. Bayer, *et al.*[5,7] The *tert*-butyloxycarbonyl (BOC)-amino acids were obtained by the pH-Stat method of Schnabel,[8] *O*-benzylthreonine according to Mizoguchi, *et al.*[9]

tert-BOC-Gly (1.75 g; 10 mmol) was allowed to react with 10 g of chloromethylated Bio-Beads SX-2, 200–400 mesh. The amount of BOC-Gly esterified to the resin was determined to be 0.35 mmol/g. Only pure, freshly distilled solvents were used for the synthesis. The total amino groups were determined by a potentiometric titration;[5] the remaining amino groups after the coupling reaction were determined *via* pyridine–HCl.[10] The yields of the coupling steps decreased from 98.6% to 96.6% ± 2%. The total amino groups remained constant, except for the last deprotection step, in which they decreased to 74% ± 3%. One cycle of the synthesis consists of the following steps: cleavage of the BOC group with 30 ml of 1 *N* HCl in acetic acid and washing with 25 ml of each of the following solvents: 2 times acetic acid, 3 times ethanol, 5 times dimethylformamide, and neutralization by reacting twice for 10 min with 30 ml of 10% triethylamine in DMF; washing 10 times with 20 ml of DMF until free of chloride and titration of the collected washing and neutralization solutions (total amino groups); washing 4 times with 25 ml of methylene chloride; coupling with a two- to fourfold excess of the BOC amino acid and the same amount of dicyclohexylcarbodiimide in 25 ml of methylene chloride; washing

(5) E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, **24**, 4353 (1968).
(6) S. Moore, D. H. Spackmann, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
(7) E. Bayer and H. Hagenmaier, *Tetrahedron Lett.*, **17**, 2037 (1968).
(8) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).
(9) T. Mizoguchi, G. Levin, D. W. Wolley, and J. M. Stewart, *J. Org. Chem.*, **33**, 903 (1968).
(10) L. C. Dorman, *Tetrahedron Lett.*, **18**, 2319 (1969).

8 times with 25 ml of methylene chloride; treatment with 25 ml of 0.3 *M* pyridine–HCl solution in methylene chloride; washing 5 times with 25 ml of methylene chloride; washing 6 times with 25 ml of DMF until free of chloride; treatment 2 times with 25 ml of 10% triethylamine in DMF; washing 6 times with DMF; potentiometric titration of the neutralization solution and washings (remaining amino groups); washing with 25 ml of the following solvents; 4 times ethanol, 2 times acetic acid. The fully protected peptide, esterified to the resin, was transesterified in a mixture of triethylamine, methanol, and hexamethylphosphoric acid triamide (1:1:6, v/v) for a period of 48 hr.[11] After a filtration and concentration 2.05 g of product (60% yield, with respect to Gly) was obtained. After gel filtration on Sephadex LH 20 in DMF (3 × 230 cm column) 735 mg of peptide (21%) resulted. Amino acid analyses were as follows: Gly, 1.06; Ala, 1.0; Val; 2.1; Leu, 1.85; Thr, 1.72 (not corrected).

The R_f values for the tlc on silica gel were: 0.6 (1-butanol–acetic acid–water, 3:1:1), 0.55 (2-butanol–pyridine–water, 2:2:1); nonuniform spots, ninhydrin negative, Cl_2/tolidine positive.

For identification by mass spectrometry[12] 31.7 mg of the product was treated for 1 hr with 1 ml of TFA, precipitated with ether, dissolved in methyl alcohol, and again precipitated with ether. The dried precipitate was acetylated for 1 hr with 3 ml of 50% acetic acid–acetic anhydride; 8.7 mg of the acetylated peptide mixture obtained after evaporation to dryness was permethylated according to the method described by Hakamori.[13] The mass spectrum of the permethylated peptide mixture was measured on the LKB 9000 (LKB Produkter Stockholm). Under the conditions employed, the peptide bond is preferentially cleaved, and the fragmentation series corresponding to cleavage of amino acids from the peptide with retention of the positive charge on the acyl fragment (*i.e.*, Ac-(CH_3) Val-(CH_3) Thr(Bzl)-(CH_3)Val- etc. for V and Ac-(CH_3) Thr(Bzl)-(CH_3)Val- etc. for VI were found.

For end group determination, 1 mg of the peptide mixture was deblocked with TFA, dansylated according to the method described by Hartley and Massey,[14] and hydrolyzed with 6 *N* HCl at 110° for 20 hr. Tlc with benzene–pyridine–acetic acid (80:20:5, v/v) on silica gel showed the presence of the derivatives of valine and threonine (R_f: 0.83 and 0.27). Estimation of the fluorescence of both spots gave a ratio of about 1:2.

The trifluoroacetylation was carried out with methyl-TFA after cleavage of the BOC group by treatment with 1 *N* HCl in acetic acid for 30 min.

Acknowledgment. We gratefully acknowledge the support of this work by the Deutsche Forschungsgemeinschaft.

(11) H. C. Beyerman, H. Hindriks, and E. W. B. De Leer, *Chem. Commun.*, 1668 (1968).
(12) E. Vilkas and E. Lederer, *Tetrahedron Lett.*, **26**, 3089 (1968).
(13) S. Hakamori, *J. Biochem. (Tokyo)*, **55**, 205 (1964).
(14) B. S. Hartley and V. Massey, *Biochim, Biophys. Acta*, **21**, 58 (1956).

Stereochemical Basis of Anticonvulsant Drug Action. II. Molecular Structure of Diazepam[1]

Arthur Camerman*[2a] and Norman Camerman[2b]

Contribution from the Department of Biological Structure, University of Washington, Seattle, Washington 98105, and the Department of Biochemistry, University of Toronto, Toronto 181, Ontario, Canada. *Received April 1, 1971*

Abstract: The crystal structure of diazepam has been determined as part of an investigation into relationships between molecular shape and pharmacological activity of anticonvulsant drugs. The compound crystallizes in the monoclinic system with cell dimensions $a = 12.928$, $b = 13.354$, $c = 7.976$ Å, $\beta = 90.01°$, space group $P2_1/a$. The structure was determined by direct centrosymmetric phasing procedures using data collected on a four-circle diffractometer. Refinement was by anisotropic full-matrix least squares to a final *R* value of 0.039. The two phenyl rings are planar and the obtuse angle between the normals to the two planes is 125°. There are conformational features very similar to certain features found in another anticonvulsant drug, diphenylhydantoin.

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one) (I) and other derivatives of 1,4-benzodiazepine were first successfully employed in medicine as tranquilizing agents. More recently, however, these drugs have displayed pharmacological properties that indicated clinical potential for the treatment of various types of epilepsy. Diazepam, in particular, has been shown to possess specific anticonvulsant activity to varying degrees against grand mal, psychomotor, and petit mal epilepsies.[3,4] Indeed, after dramatic results were obtained from clinical tests, diazepam was praised as the "drug of choice for the emergency treatment of all cases of status epilepticus."[5] The role of diazepam as a most important antianxiety agent and at the same time as a useful anticonvulsant seemingly contradicts the general rule that desynchronizing drugs are also good antiepileptics while synchronizing drugs may have a facilitating effect on seizure discharge.[6] In the light of diazepam's range of therapeutic properties we have decided to determine the three-dimensional molecular structure of this compound and compare its steric configuration to that of other chemically different anticonvulsant drugs. A preliminary account of the similarities in configuration between diphenylhydantoin and diazepam has been reported.[7]

* Department of Neurology, University of Washington, Seattle, Wash. 98105. Investigator, Howard Hughes Medical Institute.
(1) Part I of this series: A. Camerman and N. Camerman, *Acta Crystallogr., Sect. B*, in press.
(2) (a) Department of Biological Structure, University of Washington, Seattle, Washington 98105. (b) Department of Biochemistry, University of Toronto, Toronto 181, Ontario, Canada.
(3) L. O. Randall, G. A. Heise, W. Schallek, R. E. Bagdon, R. F. Banziger, A. Boris, R. A. Moe, and W. B. Abrams, *Curr. Ther. Res. Clin. Exp.*, 3, 405 (1961).
(4) For a review see: P. A. Boyer, Jr., *Dis. Nerv. Syst.*, 27, 35 (1966).
(5) H. Gastaut, R. Naquet, R. Poiré, and C. A. Tassinari, *Epilepsia*, 6, 167 (1965).
(6) R. Visioli, G. F. Ricci, L. Pastena, and L. Albani-Medolago, *Proc. Int. Congr. Neuro-Psychopharmacol., 5th*, **1966**, 1993 (1967).