The Merrifield Peptide Synthesis Studied by Near-Infrared Fourier-Transform Raman Spectroscopy

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Abstract: The stepwise solid-phase synthesis of the peptide H-(Ala)$_6$-Lys-OH on a polyacrylamide gel resin was followed by near-infrared (NIR) Fourier-transform (FT) Raman spectroscopy. In particular, this investigation involved the use of Fmoc as the N-α-protecting group. The deprotection of the Fmoc group by standard methods could be quantitatively followed. The deprotection was essentially complete until a number of alanine residues in multiples of six were reached, with nearly one-third of the peptide chains left protected after the standard piperidine treatment. Even a prolonged deprotection time did not result in a complete deprotection of the Fmoc group. This phenomenon could be attributed to the formation of secondary structures, which were indicated by structurally sensitive Raman bands, with particular focus on the amide III bands. The Fmoc group was found to have a clear influence upon the secondary structure, supporting mainly a β-sheet conformation, whereas more coiled forms were found for the deprotected samples. Preliminary studies with the use of Boc as the N-α-protecting group showed that this group had essentially no importance for the secondary structure of the pendent peptide chains. Investigation of peptides containing both D- and L-chiral forms of alanine supported the hypothesis that the presence of the Fmoc group influences the secondary structure. The swelled forms of the sequence Fmoc-(Ala)$_6$-Lys(Boc)-OR in DMSO or DMF showed different secondary structures, indicating different interactions between the peptide chains and the two solvents. Our studies show that Raman spectroscopy is a nondestructive analytical tool which allows a recording of spectra while the peptide is directly bound to the solid support under normal synthetic conditions.

Introduction

Since the introduction of stepwise solid-phase peptide synthesis by Merrifield in 1963,[1] this method has become a powerful tool in the preparation of polypeptides. The method allows the preparation of peptides with a predefined primary structure. However, important side reactions have been reported in the literature.[2] Probably the most serious problem in solid-phase peptide synthesis is the occurrence of the phenomenon termed "difficult sequences". This has been the subject of increasing attention among peptide chemists in recent years. Difficult sequences have been described in many reports.[2-23] It has been suggested that the phenomenon has its origin in the formation of a partial β-sheet structure during the chain assembly, which makes some of the amino groups inaccessible to the coupling reactions. The occurrence of such side reactions makes it important to have accurate information on yields at all steps of the peptide synthesis in order to improve the chemical method. However, when one confronts the phenomenon of difficult sequences and tries to improve the chemical methods, information about yields alone does not seem to be enough. Data from the literature implicate that the structures of the growing peptide chains influence the yields in the peptide synthesis.[4,18,21,22-34] Thus, it is important to have exact information about the structural

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behavior of the peptide chains during the synthesis. In general, when current methods are used to investigate peptide structures, results are obtained under circumstances which do not duplicate normal synthetic conditions and these methods are limited to the determination of conformations of non-resin-attached peptides or proteins.[4,25-33]

In this report we describe the use of near-infrared (NIR) Fourier-transform (FT) Raman spectroscopy as a new method for monitoring the secondary structure of the peptide chains during solid-phase peptide synthesis. Also, the present investigation shows that is is possible by NIR-FT-Raman spectroscopy to follow the deprotection yields of the N-α-protecting Fmoc group (9-fluorenylmethyloxycarbonyl) during the synthesis. The monitoring by NIR-FT-Raman spectroscopy of both the deprotection yields and the secondary structure of the peptide can be done in a noninterfering and nondestructive way under normal synthetic conditions. The present results confirm the general advantage of NIR-FT-Raman spectroscopy for analytical purposes. Preliminary conference proceedings on this subject have been given.[32,33]

Results and Discussion

We have recently identified a new problem involving an incomplete Fmoc deprotection in solid-phase peptide synthesis of homo-oligopeptides with the general formula H-(Ala)$_n$Lys-OH.[23] We found that using a standard synthetic procedure, when n = 6, resulted in 18–25% incomplete deprotection of the Fmoc group.[23,34] In the same report, we concluded that incomplete deprotection of the α-amino function and incomplete couplings have a common physical–chemical origin in the synthesis of difficult sequences. In order to obtain exact information on the structural behavior of the peptide chains during the synthesis of the above difficult sequences, we have investigated the system using NIR-FT-Raman spectroscopy.

Spectra obtained by laser excitation at 514.5 nm always resulted in a broad fluorescence band arising from unavoidable impurities. This fluorescence band was completely missing in the NIR-FT-Raman spectra recorded with excitation from the Nd/YAG laser at a wavelength of 1064 nm.

Resin and Linker. Basically two different types of solid supports are commonly used in solid-phase peptide synthesis (SPPS), polystyrene (PS) and polyacrylamide (PA). In this investigation we will focus on the use of PA as the resin derivatized with the acid-labile linker (*p*-(hydroxymethyl)phenoxy)acetic acid (HMPA). In Figure 1 is given a schematic presentation of the polymeric support PA (Figure 1A), together with the formulas for the linker (HMPA) (Figure 1B), the α-amino protecting groups *t*-Boc (Figure 1C), and Fmoc (Figure 1D).

NIR-FT-Raman spectra of the PA resin together with the resin derivatized with the linker HMPA are shown in Figure 2. The dominating band with a maximum around 1450 cm^{-1} is

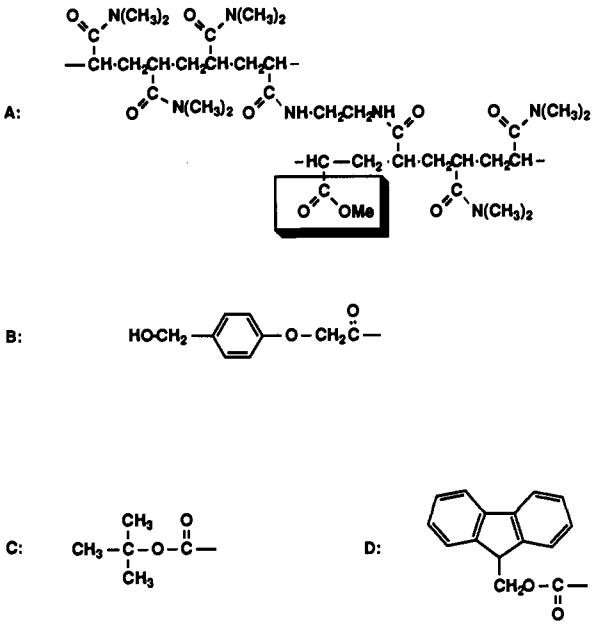

Figure 1. Schematic presentation of the PA resin (A), together with formulas for the HMPA linker (B) and protecting groups *t*-Boc (C) and Fmoc (D) used in the present investigation.

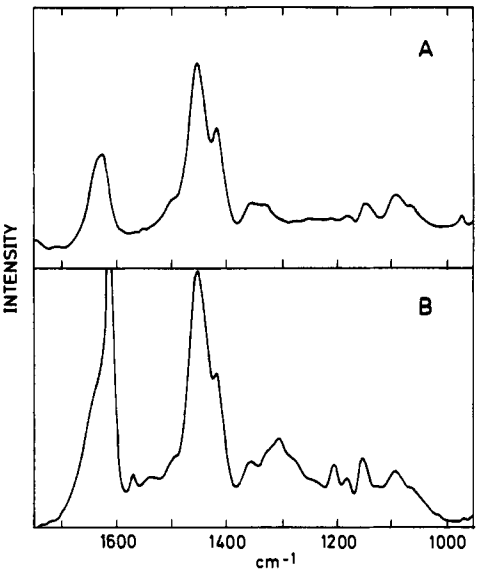

Figure 2. (A) NIR-FT-Raman spectrum of the resin (PA) and (B) spectrum of the resin derivatized with the linker.

assigned to CH_2 and CH_3 bending modes. Strong bands originating from the phenyl ring of the linker are observed at 1615 cm^{-1} and at 640 cm^{-1} (not shown). The latter is characteristic of a para-substituted benzene derivative.

The carbonyl stretching vibration of PA is observed as a rather broad band with a maximum around 1630 cm^{-1}. In this region (1600-1700 cm^{-1}) the amide I bands which are sensitive to the secondary structure of peptides and proteins are expected.[35] The carbonyl stretching vibration (1630 cm^{-1}) of the PA resin itself is observed at a lower frequency than all the structure-sensitive amide I bands (1680-1645 cm^{-1}) given by Tu.[35] This is consistent with the fact that the nitrogen-bonded hydrogen atom in ordinary peptide bonds is substituted with a methyl group in the PA resin. The carbonyl stretching band at 1730 cm^{-1} of the ester group in the linker does not interfere with the structure-sensitive amide I bands. Another peptide structure-sensitive region in the Raman spectrum is the amide III region between 1230 and 1300 cm^{-1}.[35]

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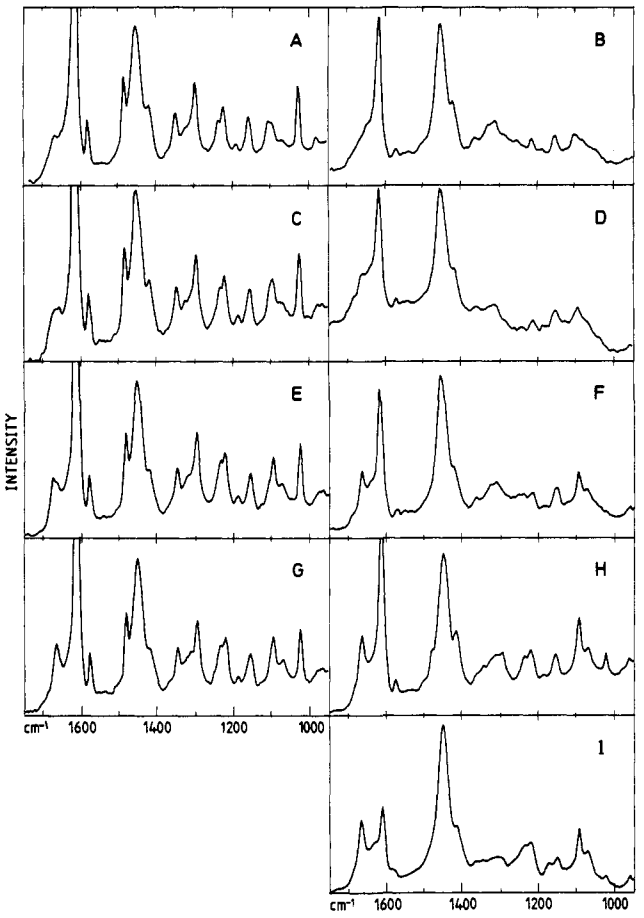

Figure 3. NIR-FT-Raman spectra obtained at each step in the solid-phase peptide synthesis of the sequences $(Ala)_n$-Lys(Boc)-OR for $n > 2$. In the left column are shown the spectra of the Fmoc-protected sequences and to the right the spectra of the corresponding deprotected sequences after a standard piperidine treatment (10 min): A and B, $n = 3$; C and D, $n = 4$; E and F, $n = 5$; G and H, $n = 6$. Spectrum I is for $n = 6$ after treatment with piperidine for 120 min.

The mode for the amide III band involves a stretching of the C–N bond and a bending of the C–N–H valence angle.[35] In this part of the spectrum, the resin–linker system shows only one rather strong band around 1220 cm^{-1} (Figure 2). This is in agreement with the fact that the amide III mode of peptides partly involves the N–H bending motion, and again the presence of the $N–CH_3$ group in the resin will shift the mode to a lower frequency as compared to the real amide III bands, which always are observed above 1230 cm^{-1}.[35] The 1220-cm^{-1} band seems a little more intense in the resin–linker system than in the resin itself, indicating that the linker might influence the structure of the resin.

Amide I Region (1600–1700 cm^{-1}). In order to investigate the structural behavior of the peptide chains during the synthesis of the peptide with the general formula H-$(Ala)_n$Lys-OR, we have obtained NIR-FT-Raman spectra at all steps during the chain assembly which include both the Fmoc-protected and the deprotected forms. Figure 3 shows spectra of resin-bound peptides with three to six alanine units. As previously mentioned, the carbonyl stretching vibrations of the PA resin are observed at frequencies lower than real amide I bands (1680–1645 cm^{-1}).[35] The carbonyl stretching band from the ester of the derivatized linker appears at Raman shifts above 1700 cm^{-1}. However, carbonyl stretching vibrations are rather broad and some overlap occurs between the peptide amide I bands and resin–linker bands in this region.

Spectra of the corresponding Fmoc-protected sequences are shown in Figure 3. No real structure-sensitive bands are observed for one or two alanine residues. Accordingly, these spectra are not shown. A weak band is observed at 1665 cm^{-1} (Figure 3). The intensity of this band increases with the length of the peptide. This is consistent with the work by Sutton and Koenig[36] investigating oligomers of alanine peptides ranging from $(Ala)_2$ to $(Ala)_6$ in the solid state. Sutton and Koenig observed a weak amide I band around 1680 cm^{-1} for the $(Ala)_2$ sequence, which was shifted to a weak band at 1659 cm^{-1} for the $(Ala)_3$ sequence. After reaching a total number of four alanine residues in the peptide, they observed the amide I band at 1663 cm^{-1}. This band was also observed for the $(Ala)_5$ and $(Ala)_6$ sequences with an increasing intensity. Unfortunately, this position of the amide I band (1665–1663 cm^{-1}) is not indicative of a particular peptide structure because this band can be assigned to either random coil, β-sheet, or β-turn.[35] However, it is possible to obtain further information about the peptide structure from the Raman bands in the amide III regions.

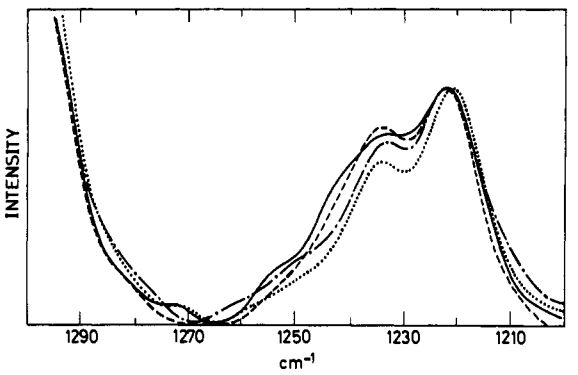

Figure 4. Detailed NIR-FT-Raman spectra in the amide III region of the resin-bound peptide sequences Fmoc-$(Ala)_n$-Lys(Boc)-OR: dotted curve, $n = 3$; dotted/broken curve, $n = 4$; broken curve, $n = 5$; full curve, $n = 6$.

Amide III Region (1230–1300 cm^{-1}). Figure 3 also includes the amide III region. In accordance with results from the amide I region, no definite structural information could be obtained with only one or two alanine residues present.

The band observed at 1222 cm^{-1} for sequences protected with the Fmoc group was changed to the region 1222–1210 cm^{-1} in the deprotected form (Figure 3). These bands do not belong to the peptide because the relative intensities are unchanged as a function of the number of alanine residues, and in accordance with this, these bands were not observed by Sutton and Koenig.[36] These bands are clearly present in the spectrum of the resin–linker (Figure 2). The shift in the frequency is most likely due to a change in the structure of the resin. Thus the Fmoc protection of the peptide may influence the resin structure.

For the Fmoc-protected sequences, a band increasing in intensity with the number of alanine residues is observed as a high-frequency shoulder on the more intense 1222-cm^{-1} band (Figure 4). The maximum for the shoulder is around 1235 cm^{-1}. According to Tu's results,[35] this band can be assigned to a β-sheet conformation consistent with our results obtained from the amide I region. Sutton and Koenig found the amide III band at 1231 cm^{-1} and concluded that the peptide was in an antiparallel β-sheet conformation.[36] The similarity between the frequencies observed for both the amide I and III bands of the Fmoc-protected peptides and for the alanine sequences given by Sutton and Koenig points toward a similarity between the secondary structures. This means that the secondary structure of the resin-bound protected peptides is of the β-sheet type. The intensity of the 1235-cm^{-1} band increases gradually for the sequences containing three to five alanine units. A small decrease is observed for the sequence containing six alanine residues. However, for this sequence, other amide III bands appear around 1245 and 1260 cm^{-1}, indicating the presence of coiled conformations.

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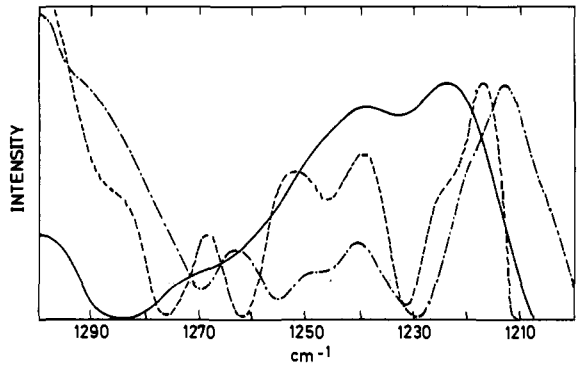

Figure 5. Amide III bands for the deprotected sequences H-(Ala)$_n$-Lys(Boc)-OR: full curve, n = 6 (after 120-min piperidine treatment); broken curve, n = 5 (after standard piperidine treatment); dotted/broken curve, n = 4 (after standard piperidine treatment).

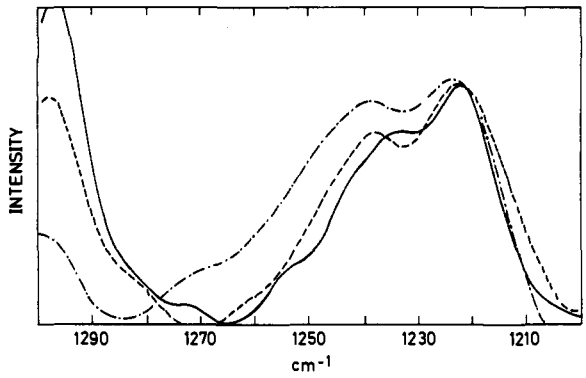

Figure 6. Amide III bands for the sequence Fmoc-(Ala)$_6$-Lys(Boc)-OR: full curve, before piperidine treatment; broken curve, after standard piperidine treatment (10 min) (30% Fmoc left); dotted/broken curve, after 120-min piperidine treatment (7% Fmoc left).

Evidently the secondary structure is influenced by the removal of the Fmoc group because the band characteristic of β-sheet conformation is no longer present after the deprotection of the Fmoc group for sequences containing up to five alanine residues (Figure 5). New bands appear at 1240 and 1263 cm^{-1} which indicate a content of β-turn and random coil in the peptide without the protecting group. Thus, it can be concluded that the secondary structure depends upon whether the peptide is protected or not. Figure 6 shows this in more detail. Results are given for the sequence containing six alanine residues before deprotection, after a standard deprotection, and after extended deprotection, corresponding to 100%, 30%, and 7% Fmoc group left, respectively. In the fully protected peptide, a β-sheet band is observed at 1235 cm^{-1}. The peptide containing 7% Fmoc is predominantly in a random coil conformation (1240 cm^{-1}) whereas the peptide containing 30% Fmoc seems to have a conformation with both some β-sheet and random coil present. Again this indicates that the peptide secondary structure is strongly dependent upon the presence of the Fmoc group. This is consistent with our chemical results[23,34] which have shown that after incorporation of the sixth alanine there are problems not only with the deprotection but also with the subsequent couplings.

Deuteration of the Peptide Resin. The vibrational mode for the amide III band exhibits considerable motion from the nitrogen-bonded hydrogen atom. Thus the amide III band is expected to be sensitive to exchange of this hydrogen by deuterium. Figure 7 shows NIR-FT-Raman spectra of the Fmoc-protected sequence containing six alanine residues, both before and after deuteration. The spectra show clearly a decrease in intensity by deuteration of the band observed at 1235 cm^{-1} relative to the band at 1220 cm^{-1}. Although the deuteration is incomplete, this change shows that the band at 1235 cm^{-1} is certainly the peptide amide III band.

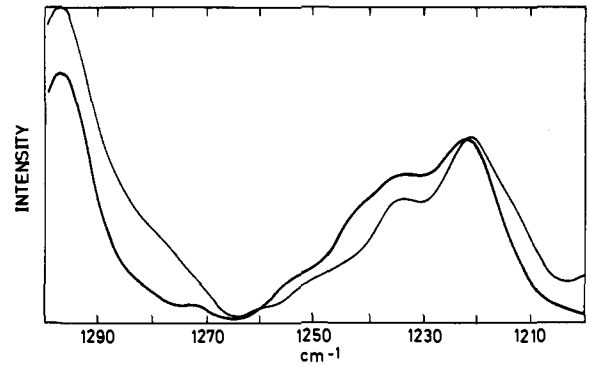

Figure 7. Amide III bands for the sequence Fmoc-(Ala)$_6$-Lys(Boc)-OR before (heavy line) and after partial deuteration of the nitrogen-bonded hydrogen atom (thin line).

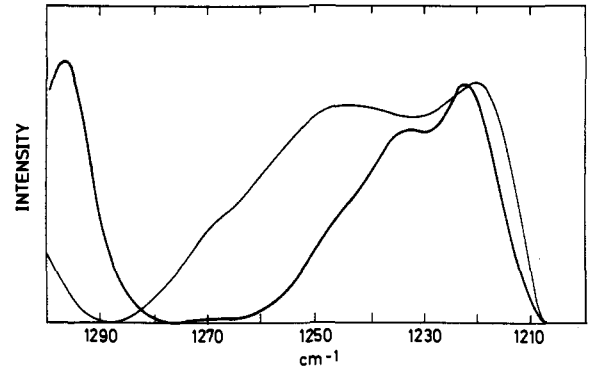

Figure 8. Amide III bands for the sequence D-Ala-(L-Ala)$_2$-D-Ala-(L-Ala)$_2$-Lys(Boc)-OR in both the Fmoc-protected (heavy line) and the deprotected form (thin line).

D-Alanine Sequences. All experiments described so far have been performed with L-alanine. In order to investigate the effect of D-alanine residues on the secondary structure, a number of experiments were performed where D-alanine residues were introduced on specific sites in the sequence containing six alanine residues. An example is shown in Figure 8 which shows the amide III region for D-Ala-(L-Ala)$_2$-D-Ala-(L-Ala)$_2$-Lys(Boc)-HMPA-PA in both protected and deprotected forms. As mentioned previously, only the amide III band is significantly sensitive to changes in the secondary structure. Thus, only this region will be discussed in context of the D-alanine-substituted peptides. The spectrum of the Fmoc-protected D-alanine sequence (Figure 8) is almost identical to the spectrum of the Fmoc-protected all-L-alanine sequence (Figure 5). The results show that the dominating β-sheet structure is formed independently of whether D- or L-alanine is present in the Fmoc-protected sequence, clearly demonstrating the influence of the Fmoc group on the secondary structure. In contrast, the deprotected D-alanine sequence is very different from the corresponding all-L form which shows content of β-sheet, β-turn, and random coil structures (Figure 4, full curve). The spectrum of the D-alanine sequence contains mainly an amide III band at 1241 cm^{-1}, indicating a high content of random coil. This is in agreement with our synthetic results, where no problems were observed in the deprotection or in the coupling reactions building up the D-alanine-containing sequences, which is in contrast to the case of the pure L-alanine sequences. Merrifield et al. have obtained similar results.[18] They found it almost impossible to build up pure L-alanine sequences, but substitution of L-alanine with the D form in the above mentioned positions totally eliminated the synthetic problems.

Boc Protection vs Fmoc. In order to investigate further the hypothesis that the Fmoc group may promote β-sheet conformation, we have synthesized the alanine sequence protected with the Boc group on the N-terminal (Boc-(Ala)$_6$-Lys(Boc)-OR).

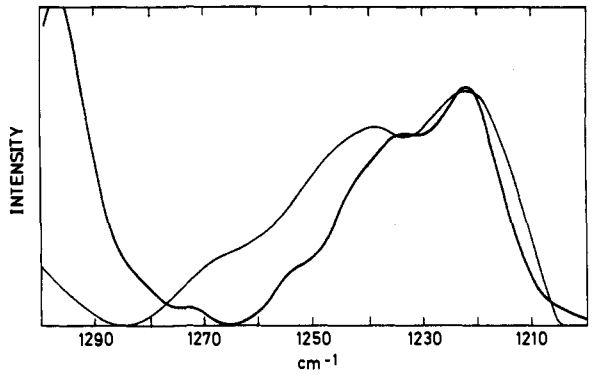

Figure 9. Amide III bands for the protected sequence -(Ala)$_6$-Lys(Boc)-OR. The heavy line shows the Fmoc-protected whereas the thin line shows the Boc-protected sequence.

Figure 9 shows the NIR-FT-Raman spectrum of the Boc-protected peptide resin. The spectrum exhibits a dominating amide III band at 1240 cm^{-1}, which shows that, to a high degree, the peptide chains in the Boc-protected form are in a random coil conformation. Thus, the Fmoc protecting group significantly influences the conformation of the peptide chains, resulting in an increased cross-linking of the resin.

Deprotection Studied by Characteristic Fmoc Bands

Current methods to investigate yields in intermediate stages of peptide synthesis are numerous. Quantitative determinations of amino components have been done by titration or uptake of different reagents,[37-40] by ninhydrin analysis,[41-42] and by displacement of a colored aldehyde from its Schiff base[43] or of other chromophoric adducts.[44,45] Measurement of deletion peptides has been performed by a preview sequencing of the peptide resin,[46] by mass spectrometry,[47,48] and by different separation techniques on the product after cleavage of the peptide from the solid support.[23,34,49-51] Some of these methods are very sensitive. For example, the quantitative ninhydrin reaction[42] gives reliable data about uncoupled amino groups down to less than 0.1%. Recently, Merrifield et al. described a very sensitive method for measuring deletion peptides[18] (0.02% per cycle) using ^{252}Cf fission fragment ionization time-of-flight mass spectrometry. However, most techniques require additional reactions to introduce and remove a reporter reagent. Furthermore, a withdrawal of a sample of the peptide resin for testing is usually necessary. Some attempts have been made to develop techniques which allow a continuous real-time monitoring of the acylation reaction. Sheppard et al. have reported the use of colored ester derivatives,[45,52] and Krchnak et al. have reported the use of bromophenol blue.[53] These methods rely on acid–base indicators which generate colored anions in the proximity of unreacted amino groups on the solid support. Thus, the quantization requires a translucent support. It has been reported that arginine (Arg) and histidine (His) can cause coloration of fully acylated reaction mixtures.[54] Young et al. have reported the use of an anionic dye in counterion distribution monitoring.[54] However, using this method, similar problems have been observed with Arg and His, which, due to the basicity of the side chains, can participate in an unspecified binding of the dye to the solid support.[55]

As an alternative to free amino groups, coupling reactions can be noninvasively monitored on the basis of the properties of the UV absorption of the Fmoc group at 304 nm.[56] Unfortunately, this method lacks the sensitivity to give accurate information about coupling yields. The same low-sensitivity problem is encountered using this method to follow the deprotection of the Fmoc group.

Most of the monitoring methods mentioned above seem to lose their sensitivity in the case of difficult sequences in solid-phase peptide synthesis. For example, the dye or the receptor reagents need to be in close contact with amino groups, which in the first place have avoided reaction with an activated amino acid. Furthermore, the ninhydrin test, which works at elevated temperature, has been shown to give poor results[27] when used in a synthesis involving difficult sequences. Thus, the field of monitoring peptide synthesis clearly needs to be supplemented and improved.

Raman spectra of the resin-bound Fmoc-protected homo-oligopeptides are included in Figure 3. The Fmoc group contains an aromatic system which gives rise to strong bands in the Raman spectrum. The ring stretching vibration at 1615 cm^{-1} of the fluorenyl system in the Fmoc group overlaps with the stretching vibration of the para-substituted phenyl ring from the linker (HMPA). In fact, the two bands merge into one strong band at 1615 cm^{-1}. Other characteristic bands for the fluorenyl system of the Fmoc group are observed at 1297 and 1025 cm^{-1}, in accordance with expectations for ortho-substituted benzene rings. These Fmoc bands are easily assigned by comparison of corresponding peptides both with and without the protection group (Figure 3). The comparison leads to an assignment of the band observed at 1480 cm^{-1} as originating from the Fmoc group.

As described in previous work,[23,34] in some cases the Fmoc group is not completely removed by standard treatment with piperidine. Figure 3 shows that small amounts of the Fmoc group are left in the deprotected samples because bands are still observed from the fluorenyl system at 1480, 1297, and 1025 cm^{-1}. This incomplete deprotection is of crucial importance for the solid-phase synthesis of peptides; thus it is important to develop a quantitative method to follow the deprotection reaction. In order to do this, we have compared the intensity of the Fmoc band at 1025 cm^{-1} to the intensity of the CH_3 and CH_2 bands around 1450 cm^{-1}. A sharp band in the protected molecules at 1480 cm^{-1} has been assigned to the Fmoc group. This band must be caused by either a ring vibration or the bending of the methylene group in the Fmoc group. The shape of the remaining band from 1400 to 1500 cm^{-1} does not change noticeably from the protected to the deprotected forms of a given peptide. This might support

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the assignment of the Fmoc methylene group to the 1480-cm^{-1} band. Even if this is false, the similarity between the broad, strong features of the methylene bendings of the protected and the deprotected forms in comparison with the rest of the peptide resin ensures that the Fmoc methylene group does not influence the total integrated intensity of this band. Thus the band at 1450 cm^{-1} can be taken as an intensity standard against which the remaining Fmoc groups can be quantified after the deprotection process. The methyl group in the side chain of alanine residues could contribute to the band at 1450 cm^{-1}. In order to eliminate the influence of this CH_3 group, comparisons were only performed between protected and deprotected sequences which contained the same number of alanine residues. The removal of the Fmoc protection group is very effective for sequences containing up to five alanine residues, where approximately 1% Fmoc was found in the deprotected samples. This percentage is close to the sensitivity of the method at the present stage. However, improvements could be introduced by computational methods. For sequences containing six alanine residues, the deprotection is very ineffective, with 30% remaining after the standard piperidine treatment. Even after prolonged deprotection time (120 min), 7% of the Fmoc groups remain deprotected (Figure 3). These values are in very good agreement with results from standard methods used for this quantization.[23,34] However, these methods all include detaching the peptide from the resin, isolation and lyophilization, and finally HPLC for the estimation and identification. All these procedures are very tedious and indeed sensitive to unavoidable impurities. Using the NIR-FT-Raman method, the deprotection of the N-terminal can be followed directly during the synthesis of the peptide.

The quantitative results could certainly be improved by sophisticated computer programs. More bands originating from the Fmoc group, i.e. 1297 and 1470 cm^{-1}, could be included. Although these bands show more overlap with other bands in the spectrum, a procedure involving more bands than the band at 1025 cm^{-1} due to the ortho substitution should easily improve the quantitative method to a sensitivity less than 1%.

Structural Behavior of Swelled Peptide Resins

It has been shown that peptide resins swell to different volumes in different solvents.[57] Concerning difficult sequences, these results indicate that aggregation of the peptide chains can be overcome to some extent by using the right solvent. Throughout the synthesis of the above mentioned sequences, we have used DMF as the solvent. In order to investigate the influence of swelling on the structural behavior of the peptide chains, we have obtained NIR-FT-Raman spectra of the sequence Fmoc-(Ala)$_6$-Lys(Boc)-OR swelled both in DMF and in DMSO. The results are shown in Figure 10. The spectra of the swelled peptide resin seem almost identical to the spectra obtained for the dry form. More detailed spectra of the amide III region (Figure 11) show that the structure of the peptide chains has not changed from the dry form after swelling in DMSO.

In contrast, some structural changes have occurred during the swelling in DMF. The band around 1235 cm^{-1} has decreased in intensity, and the maximum has shifted slightly to 1237 cm^{-1}. Furthermore, a new band has appeared at 1255 cm^{-1} (Figure 11). These results indicate that a conformational change from a β-sheet to a β-turn and random coil takes place upon swelling in DMF, most likely because DMF is able to break parts of the hydrogen-bonded peptide aggregates. The differences in swelling power of solvents in SPPS show that the swelling power of a given solvent depends on the peptide sequence. These experiments show that information about the peptide structure and deprotection yields can easily be obtained from Raman spectra of the swelled peptide resin during the synthesis.

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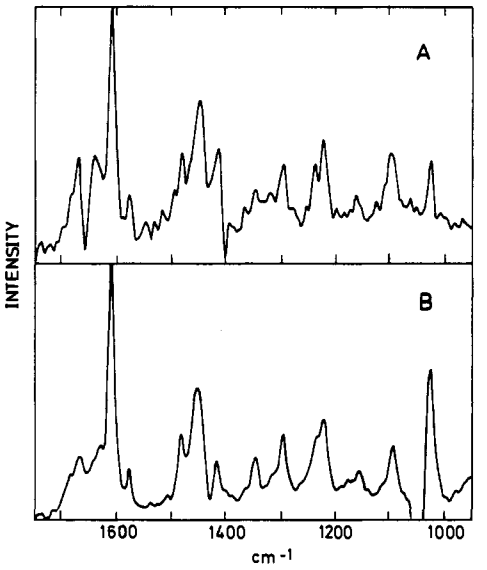

Figure 10. Raman spectra of the sequence Fmoc-(Ala)$_6$-Lys(Boc)-OR swelled in DMF (A) and in DMSO (B).

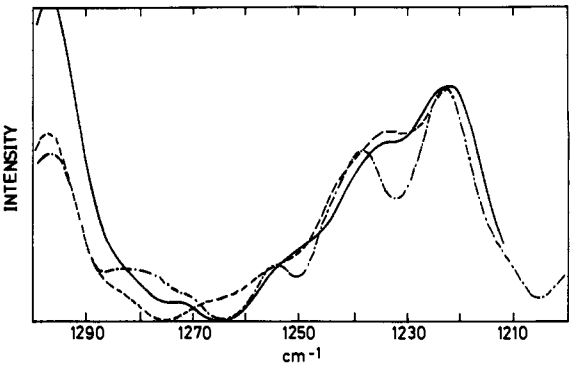

Figure 11. Amide III bands for the sequence Fmoc-(Ala)$_6$-Lys(Boc)-OR in dry form (full line), swelled in DMF (dotted/broken line), and swelled in DMSO (broken line).

Conclusion

Although Raman spectroscopy is a nondestructive analytical tool without any special sample preparation, the general use of this method with excitation in the visible part of the electromagnetic spectrum is very often prevented by the competitive fluorescence mechanism arising from unavoidable impurities. The present investigation, in accordance with a number of recent results,[58,59] shows that this problem is solved by the use of an exciting laser wavelength in the NIR region.

The solid-phase peptide synthesis can be monitored in a nondestructive way by the use of NIR-FT-Raman spectroscopy. In this investigation, the synthesis of the peptide H-(Ala)$_6$-Lys-OH on a polyacrylamide gel resin was followed. A main advantage of the NIR-FT-Raman method is that spectra of the resin-bound peptide were obtained directly.

The use of Fmoc as a N-α-protecting group was studied in detail. Deprotection by standard methods was efficient until a total number of six alanine residues was reached, whereupon a remarkable decrease in the efficiency of the deprotection of the Fmoc group was observed. This phenomenon might be due to specific secondary structures and cross-linking between different peptide chains.

The amide I and amide III bands were used in the study of secondary structures. Amide III bands were shown to be real

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structure-sensitive bands by partial substitution of the nitrogen-bonded hydrogen with deuterium. Amide III bands were particularly useful in studies of secondary structures. The results revealed that the homo-oligopeptide secondary structure for a given number of alanine units was strongly influenced by the Fmoc protecting group. In general, the presence of this group supports a β-sheet structure, whereas the deprotected samples seem to contain a higher content of coil structures. Preliminary experiments showed that Boc protection of the α-amino group did not provoke the tendency toward β-sheet formation in the protected peptides. Thus, the Fmoc protecting group influences the peptide secondary structures by including cross-linking between the resin-bound peptides. This was further confirmed by studies of Fmoc-protected and deprotected alanine sequences containing both D- and L-chiral forms.

The secondary structures of the Fmoc-protected sequence containing six alanine residues swelled in either DMSO or DMF were easily followed. Different effects on the peptide conformation were observed for the two solvents.

The use of NIR-FT-Raman spectroscopy in SPPS is very promising. Other resins, sequences, handles, and different protection strategies need to be investigated. Further study might reveal a close relationship between secondary structures and the problems found in the synthesis of difficult sequences. Use of fiber optics to obtain Raman spectra of samples at remote locations might even indicate a nondestructive and real-time monitoring of solid-phase peptide synthesis on an industrial scale.

Experimental Section

NIR-FT-Raman Spectra. The NIR-FT-Raman spectra were obtained on a Bruker IFS-66 FTIR spectrometer equipped with a FRA-106 Raman module and a cooled Ge detector. A Nd/YAG laser was used as the exciting source (1064 nm) with an output around 300 mW. The 180°-scattering configuration was used. Quartz tubes with an inner diameter of 2-4 mm were used as sample cells.

Peptide Synthesis. The resin-bound peptide H-(Ala)$_n$Lys(Boc)-OR was synthesized on a polyacrylamide gel resin (PepSyn Gel resin 1.0 mmol/g) with a HMPA linker. The first amino acid was coupled as a symmetrical anhydride in DMF, and the following amino acids were coupled as Fmoc-protected Dhbt esters in DMF. The deprotection of the Fmoc group was carried out under standard conditions by 20% piperidine in DMF (10 min). Detailed information about the peptide synthesis, purification, and identification is published elsewhere.[34]

Swelling Experiments. A 10-mg sample of peptide resin was swelled in DMF or DMSO for 1 h and then washed three times with the solvent before obtaining the Raman spectrum.

Deuteration of Fmoc-(Ala)$_6$Lys(Boc)-OR. A 10-mg sample of peptide resin was treated with deuterated acetic acid for 1 h under gentle agitation before obtaining the Raman spectrum.

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