Journal of Peptide Science
J. Peptide Sci. **4**: 461–470 (1998)

Monitoring of Solid Phase Peptide Synthesis by FT-IR Spectroscopy

BERND HENKEL and ERNST BAYER*

Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

Received 7 March 1998
Accepted 13 April 1998

Abstract: Aggregation phenomena of growing peptides on the resin have seldom been investigated. We report here how conformations are determined by FT-IR spectroscopy. Therefore the sequence 80–99 of HIV 1-protease was synthesized. After every coupling a resin sample was taken out of the reaction column and a FT-IR spectrum recorded. The results were compared with the UV monitoring obtained from another synthesis of the same peptide. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: monitoring; solid phase peptide synthesis; FT-IR spectroscopy; difficult sequences; aggregation

INTRODUCTION

Solid-phase peptide synthesis has become a powerful tool in the preparation of peptides since its introduction by Merrifield [1]. A major problem that has to be dealt with during the polypeptide synthesis is the occurrence of the so-called 'difficult sequences' [2,3], due to the intra-chain aggregation phenomena of the growing peptide chain. There are some remedies reported [4–12] to overcome these problems.

The early determination of secondary structures which might lead to dramatic decreases in coupling yields is important for the fact that coupling conditions can be changed to prevent poor couplings. UV monitoring, conductivity-monitoring and swellographic monitoring [13–16] only indicate if a synthesis is already bad and give no early warning. However, FT-IR [17–27] and FT Raman spectroscopy [28,29] might indicate and predict synthesis difficulties several coupling steps ahead.

There are mainly three significant infrared absorption bands in peptides and proteins: (1) the amide A band (~ 3300 cm^{-1}) which characterizes the N–H-stretching mode; (2) the amide I band which is caused by C=O-strecthing mode (~ 1650 cm^{-1}); (3) the amide II band which comes from the N–H-bending mode (1520–1550 cm^{-1}). The IR absorptions of these bands are strongly influenced by the formation of secondary structures occurring during peptide synthesis on the solid phase.

The investigation of TentaGel-bound peptides [30] with FT-IR spectroscopy has been reported. For this reason the sequence 80–99 of HIV 1-protease was synthesized. Starting with coupling step No. 7 a resin-sample was taken out of the reaction column of the Milligen 9050 continuous flow peptide synthesizer and was subjected to FT-IR measurement after every coupling. Special attention was paid to the amide I and II region. The results obtained were compared with the UV monitoring of the synthesis of this peptide.

Abbreviations: DIC, diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; FT, Fourier transform; HIV, human immunodeficiency virus; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl.

* Correspondence to: Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany.

CCC 1075–2617/98/080461-10$17.50

MATERIAL AND METHODS

General

Fmoc-amino acids (side-chain protection: *t*Bu for Thr; Trt for Asn, Cys, Gln; Mtr for Arg) and HOBt were obtained from Nova Biochem, Läufelingen, Switzerland, TentaGel resin was obtained from Rapp-Polymere, Tübingen, Germany. All other chemicals were purchased either from Merck, Darmstadt, Germany, Fluka, Buchs, Switzerland or Aldrich, Steinheim, Germany. The linker 3-methoxy-4-hydroxymethylphenoxyacetic acid was prepared by Sheppard's method [31].

Peptide synthesis was carried out on the Milligen 9050 continuous flow peptide synthesizer, FT-IR spectra were recorded with a spectrometer from Bruker (Bruker IFS 48).

Table 1 Characteristic Infrared Bands of the Amide I and Amide II Region [17–27,30]

Conformation	Amide I frequencies (cm^{-1})	Amide II frequencies (cm^{-1})
Random coil	~1655 (s)	1520–1545 (s)
β-sheet	1685 (w), 1630 (s)	1525–1530 (s)
α-helix	~1650 (s)	1540–1545 (s)

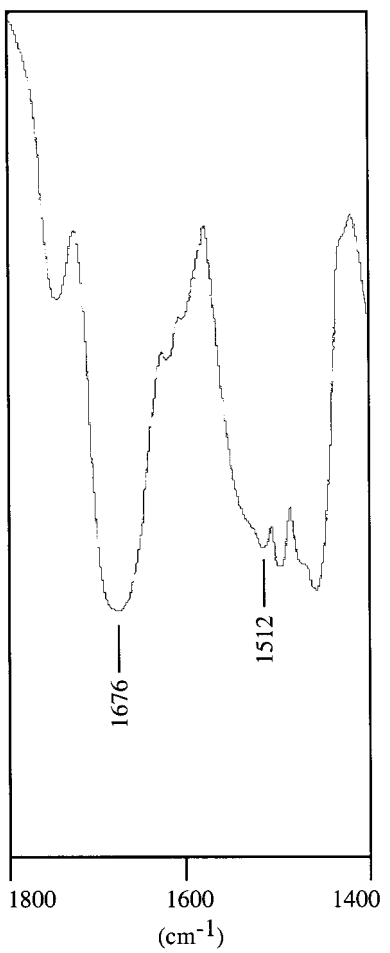

Figure 1 Coupling No. 7 (Ile): The band at 1632 cm^{-1} is symmetrical, no conformation is detected.

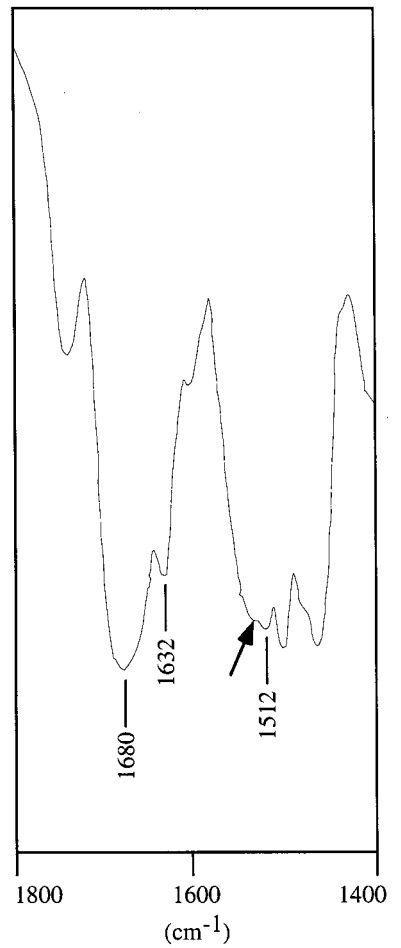

Figure 2 Coupling No. 8 (Gln): A band at 1632 cm^{-1} arises, the absorption maximum of the carbonyl-band is shifted to 1680 cm^{-1}; the band at 1512 cm^{-1} shows a little shoulder at the left hand (indicated by an arrow).

Peptide Synthesis and Resin Sampling

The linker 3-methoxy-4-hydroxymethylphenoxy-acetic acid was coupled to the TentaGel resin (0.23

mmol/g) according to the common procedure with the aid of HOBt/DIC. Fmoc-Phe-OH was coupled to the linker in a four-fold excess with equimolar amounts of DIC and HOBt and catalytic amounts of DMAP which resulted in a loading of 0.19 mmol/g. The sequence 80–99 of HIV 1-protease (H-Thr-Pro-Val-Asn-Ile-Ile-Gly-ArgA-sn-Leu-Leu-Thr-Gln-Ile-Gly-Cys-Thr-Leu-Asn-Phe-OH) was synthesized with six equivalents of HOBt/DIC for the activation and DMF as a solvent. The following protocol was used for the 1 g-scale for small resin sampling after the indicated couplings:

1. Deprotection with 20% piperidine/DMF (4 min, flow-rate 10 ml/min).
2. Wash with DMF (3 min, flow-rate 20 ml/min).

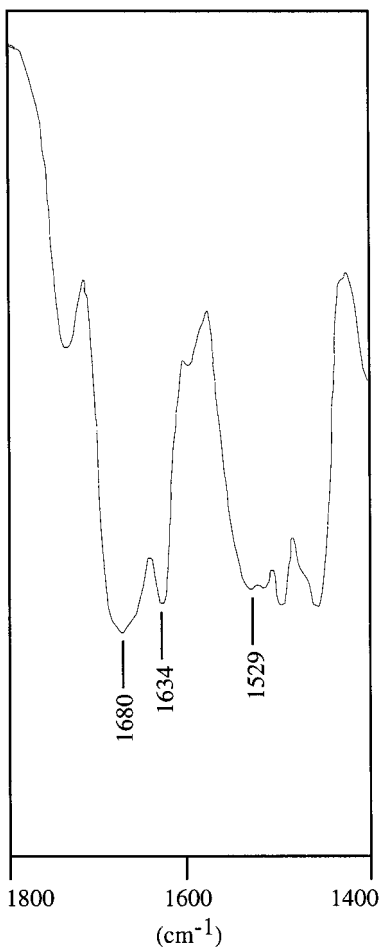

Figure 3 Coupling No. 9 (Thr): Both amide I bands (1680 cm^{-1}, 1634 cm^{-1}) and amide II band indicate the formation of a β-sheet.

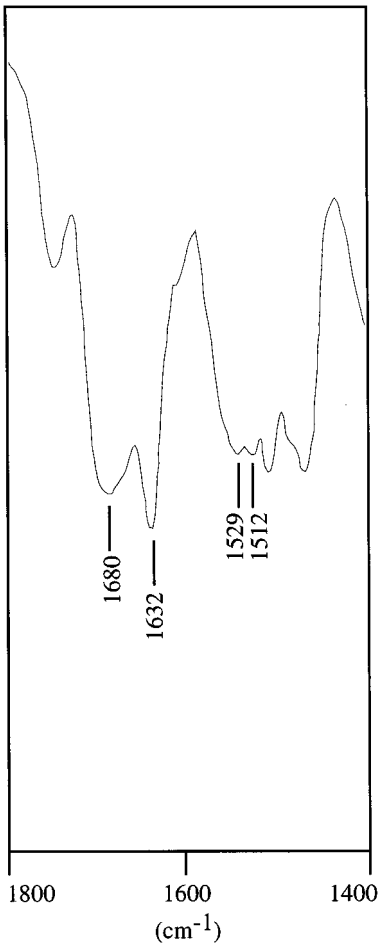

Figure 4 Coupling No. 10 (Leu): β-sheet is dominant, band at 1632 cm^{-1} gets stronger.

3. Activation of the Fmoc-amino acid during step 1 and 2 (six-fold excess of Fmoc-amino acid, HOBt and DIC in 2 ml DMF for 10 min).
4. Coupling (15 min, flow-rate 20 ml/min).
5. Re-coupling for the amino acid derivatives Fmoc-Ile-OH and Fmoc-Arg(Mtr)-OH.
6. Wash with DMF (3 min, flow-rate 20 ml/min).
7. Sampling of resin with the intact Fmoc-group starting after coupling step No. 7 (Ile) until coupling step No. 20 (Thr).

Preparation of the KBr Pellets

The resin samples were washed thoroughly with DMF, methanol and diethyl ether and dried under vacuum. Each sample was mixed with KBr to form a pellet. These pellets were subjected to FT-IR mea-

surements. The resin samples were not weighed before preparing the pellets.

RESULTS AND DISCUSSION

Many correlations [17–27,30] have been established between the characteristic infrared bands of peptides and their conformations (Table 1).

β-sheets, α-helices and random coil structures can exist in one peptide. When the conformation changes during the peptide synthesis IR absorptions can shift to one direction or the other. The occurrence of a β-sheet can be clearly determined by the splitting up of the amide I band. There is no difference between a FT-IR spectrum of the KBr pellet with the peptide containing solid support and the resin in the swollen state [29]. In Figures 1–14 the FT-IR monitoring of the synthesis of fragment 80–99 of HIV 1 protease is shown.

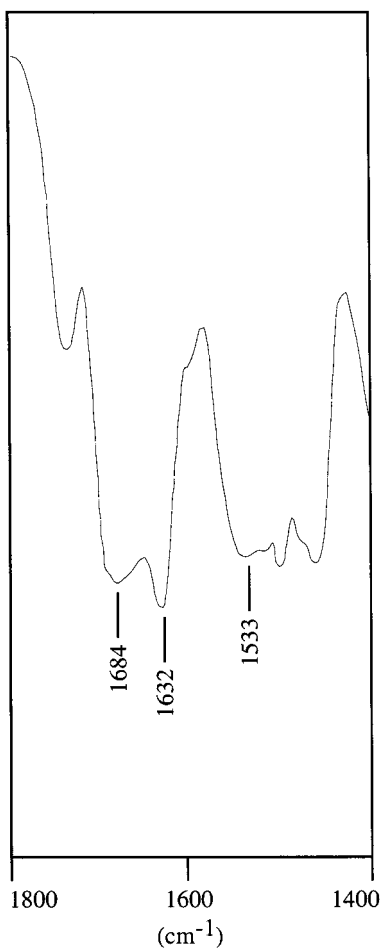

Figure 5 Coupling No. 11 (Leu): β-sheet remains dominant.

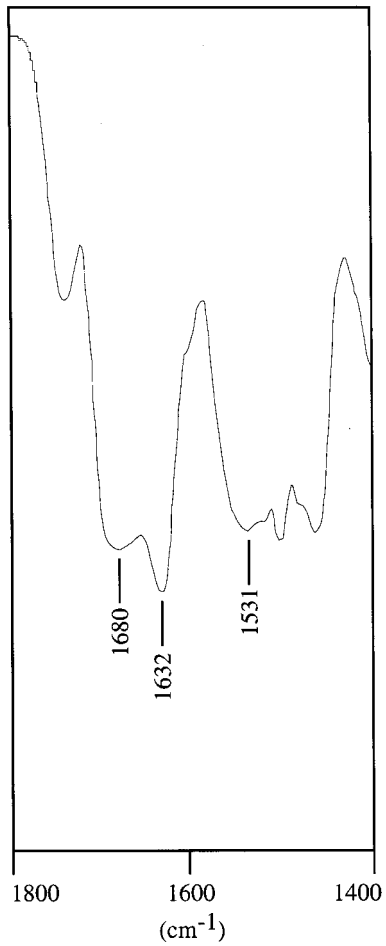

Figure 6 Coupling No. 12 (Asn): Band at 1680 cm^{-1} is less pronounced, β-sheet is dominant.

The FT-IR measurements clearly show the onset of a β-sheet after coupling No. 8 (Gln) whereas no smaller deprotection peak can be seen from the UV monitoring. The β-sheet structure gradually becomes stronger and reaches its maximum at coupling No. 13 (Arg) or coupling No. 14 (Gly). Starting with coupling No. 13 (Arg) more and more random coil portions were detected while a β-sheet is still present. This is in contrast to the UV monitoring of the Fmoc-deprotection (Figure 15) by which incomplete couplings caused by conformational changes can be seen not until position No. 10 (Leu), FT-IR detected the tendency to form a β-sheet already at position No. 8 (Gln). The coupling yield decreases dramatically at position No. 12 (Asn) followed by a

gradual increase due to the onset of random coil structure.

The increase of the deprotection peaks during the further synthesis results from the gradual accessibility of final amino groups of truncated TentaGel-bound peptides by activated Fmoc-amino acids which leads to failure sequences.

CONCLUSIONS

FT-IR spectroscopy offers a viable option for the determination of conformations of polymer-bound peptides. In contrast to the UV monitoring the tendency to form a β-sheet can be predicted. FT-IR spectroscopy already shows the onset of a β-sheet at coupling No. 8 (Gln), whereas nothing can be seen from the UV monitoring until the β-sheet becomes dominant at coupling No. 11 (Leu). Comparative studies of the resin-samples after each coupling with FT-IR spectroscopy facilitate the identification of 'difficult sequences' before they lead to poor deprotections or coupling yields. Consequently, precautions can be taken to avoid the formation of such a β-sheet in the coupling steps followed in the peptide assembly.

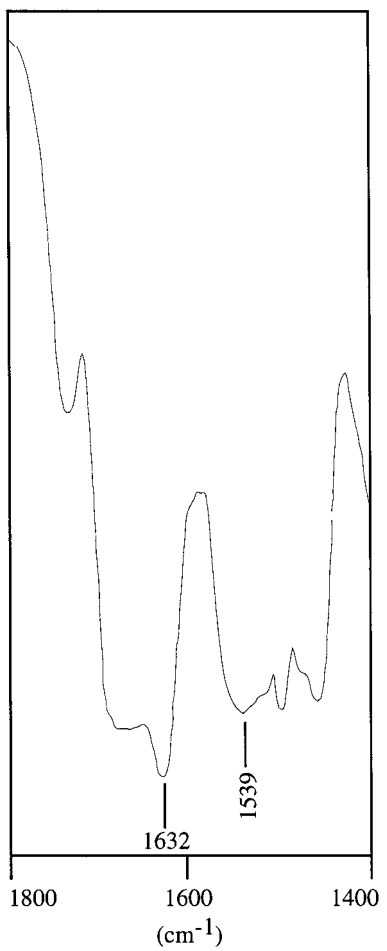

Figure 7 Coupling No. 13 (Arg): Double structure of amide I band has nearly dissappeared, amide II band shows random coil portions (1539 cm^{-1}).

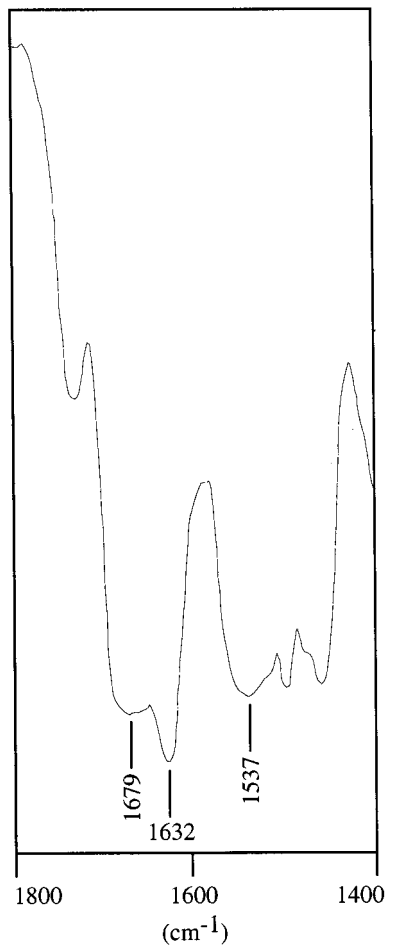

Figure 8 Coupling No. 14 (Gly): β-sheet (1679 cm^{-1}) and random coil portions (1537 cm^{-1}).

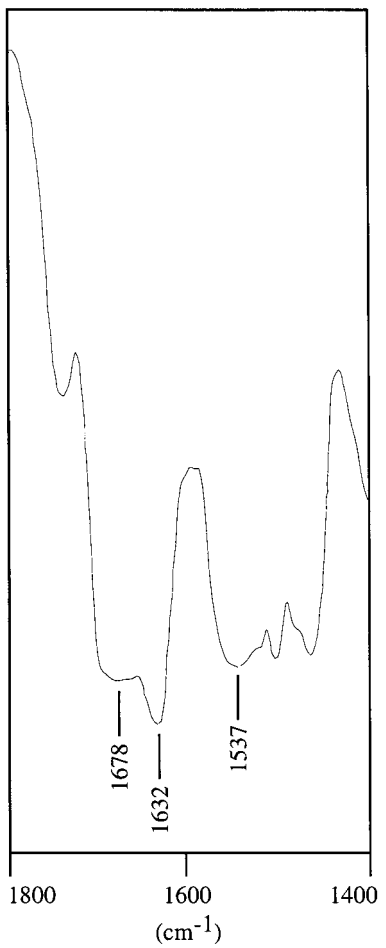

Figure 9 Coupling No. 15 (Ile): Dominant β-sheet (1678 cm^{-1}) and gradually more random coil portions observed.

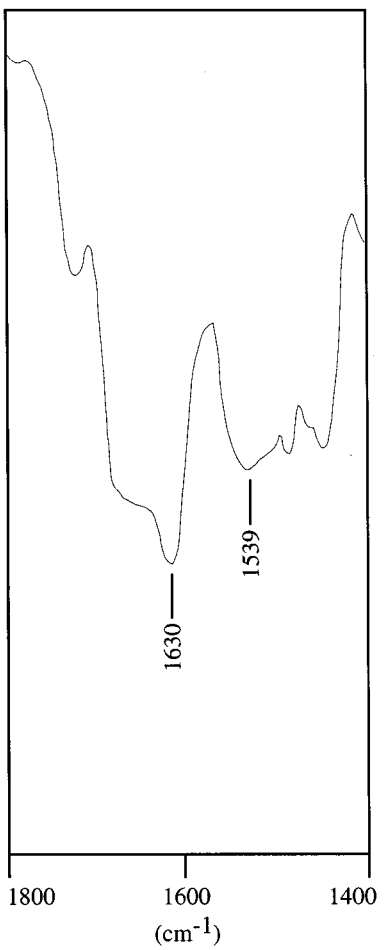

Figure 10 Coupling No. 16 (Ile): Random coil structure gets stronger (1539 cm^{-1}).

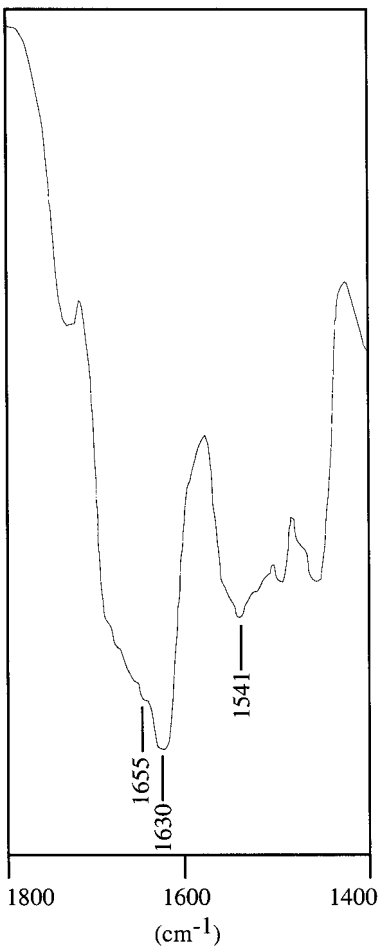

Figure 11 Coupling No. 17 (Asn): Random coil structure (1655 cm^{-1}, amide I and 1541 cm^{-1}, amide II), β-sheet still present (1630 cm^{-1}).

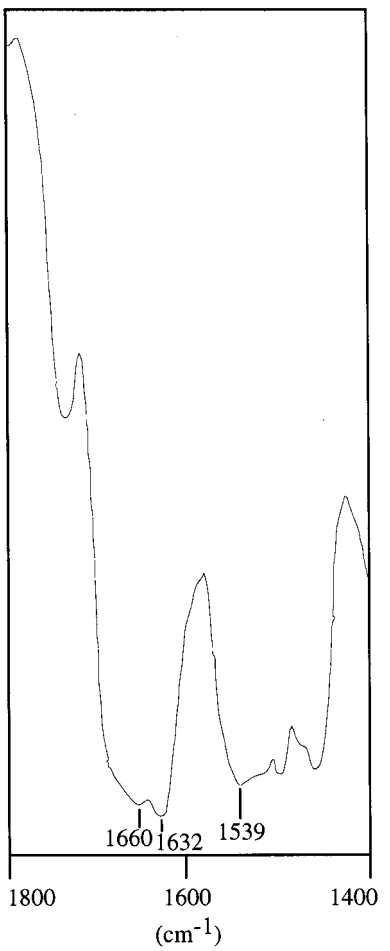

Figure 12 Coupling No. 18 (Val): Amide I band shows absorption at 1660 cm^{-1} which indicates random coil structure, amide II band also shows random coil structure (1539 cm^{-1}), β-sheet is still present (1632 cm^{-1}).

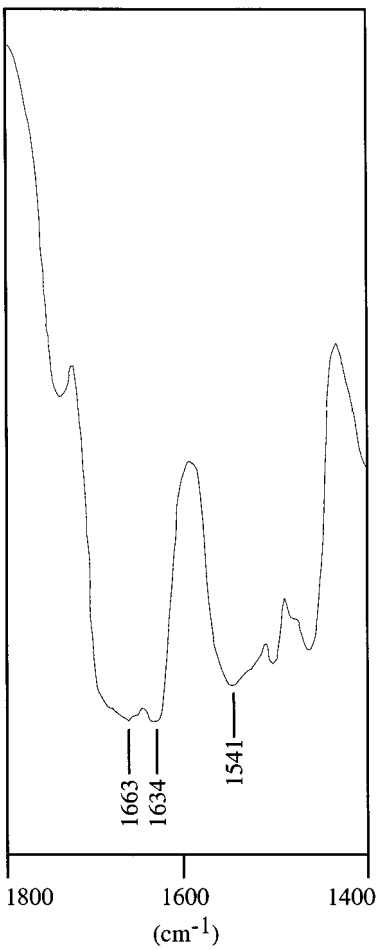

Figure 13 Coupling No. 19 (Pro): Random coil structure (1663 cm^{-1} and 1541 cm^{-1}), β-sheet is still present (1634 cm^{-1}).

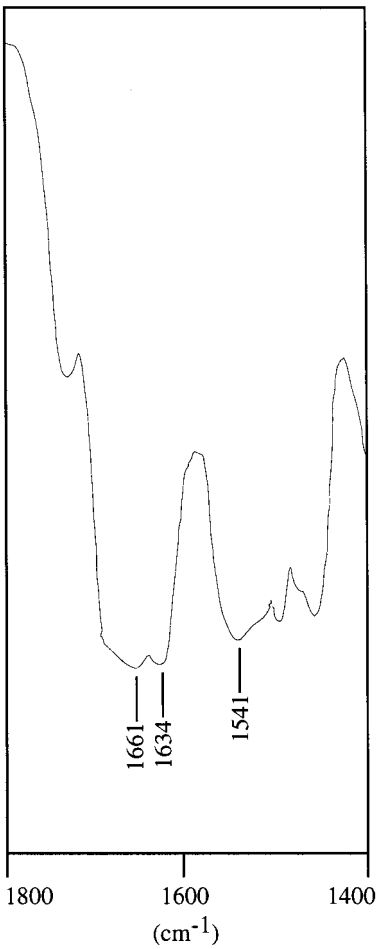

Figure 14 Coupling No. 20 (Thr): Random coil structure (1661 cm^{-1} and 1541 cm^{-1}), β-sheet is still present (1634 cm^{-1}).

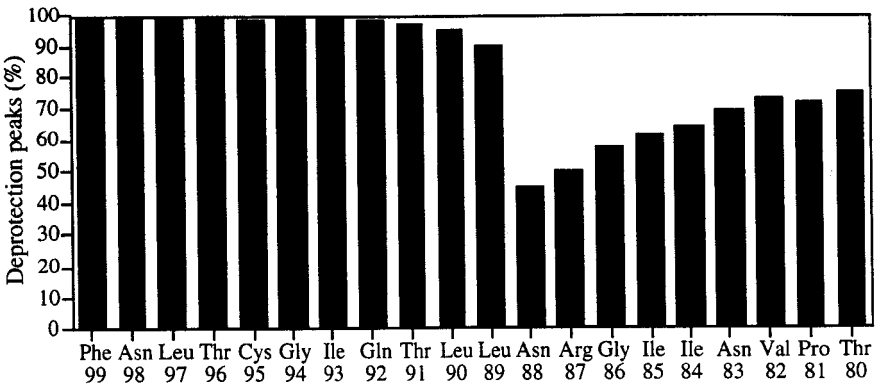

Figure 15 UV monitoring of the deprotection peaks of sequence 80–99 of HIV 1-protease.

REFERENCES

1. R.B. Merrifield (1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc. 85*, 2149–2154.
2. S.B.H. Kent, D. Alewood, P. Alewood, M. Baca, A. Jones and M. Schnolzer in: *Innovations and Perspectives in Solid Phase Synthesis*, R. Epton, Ed., p. 1–27, Intercept, Andover 1992.
3. J. Bedford, C. Hyde, T. Johnson, W. Jun, D. Owen, M. Quibell and R.C. Sheppard (1992). Amino acid structure and 'difficult sequences' in solid phase peptide synthesis. *Int. J. Peptide Protein Res. 40*, 300–307.
4. B. Henkel, Totalsynthe der HIV 1-Protease, Synthese von Peptiden und Einführang einer neuen Aukerverbindung in die Peptidsynthese, Ph.D. thesis, University of Tübingen, 1995.
5. E. Bayer (1991). Auf dem Weg zur chemischen Synthese von Proteinen. *Angew. Chem. 103*, 117–133. Protein synthesis. *Angew. Chem. Int. Ed. Engl. 30*, 113–129.
6. M. Narita, S. Isokawa, S. Honda, H. Umeyama, H. Kakei and S. Obana (1989). Individuality of amino acid residues in protected peptides. Conformational and β-sheet structure-disrupted behaviors of resin-bound peptides. *Bull. Chem. Soc. Jpn. 62*, 773–779.
7. J. M. Stewart and W. A. Klis in: *Innovation and Perspectives in Solid Phase Synthesis*, R. Epton, Ed., p. 1–9, SPCC (UK) Ltd., Birmingham 1990.
8. T. Haack and M. Mutter (1992). Serine derived oxazolidines as secondary structure disrupting, solubilizing building block in peptide synthesis. *Tetrahedron Lett. 33*, 1589–1592.
9. C. Hyde, T. Johnson, D. Owen, M. Quibell and R.C. Sheppard (1994). Some 'difficult sequences' made easy. *Int. J. Peptide Protein Res. 43*, 431–440.
10. T. Johnson, M. Quibell, D. Owen and R.C. Sheppard (1993). A reversible protecting group for the amide bond in peptides. Use in the synthesis of 'difficult sequences'. *J. Chem. Soc. Chem. Commun. 4*, 369–372.
11. N. Clausen, C. Goldammer, K. Jauch and E. Bayer in: *Peptides, Chemistry, Structure, and Biology*, P.T.P. Kaumaya, R.S. Hodges, Eds., p. 71–72, Mayflower Scientific Ltd., England 1996.
12. L. Zhang, C. Goldammer, B. Henkel, F. Zübl, G. Panhaus, G. Jung and E. Bayer in: *Innovations and Perspectives in Solid Phase Synthesis*, R. Epton, Ed., p. 711–716, Mayflower Worldwide, Oxford, England 1994.
13. Manual of Peptide Synthesis System Pioneer™ from PerSeptive Biosystems (Online UV monitoring), Niesbaden, Germany.
14. Manual of Applied Biosystems Model 433 A Peptide Synthesizer from Applied Biosystems (UV monitoring and conductivity monitoring), Weiterstadt, Germany.
15. I.L. Rodionov, I.A. Peshenko, L.G. Mustaeva and V.T. Ivanov in: *Innovation and Perspectives in Solid Phase Synthesis*, R. Epton, Ed., p. 665–668, Mayflower Worldwide, Oxford, England 1994.
16. L. Rodionova, I. Rodionov, L. Bydakova and V.T. Ivanov in: *Peptides 1994*, H.L.S. Maia, Ed., p. 169–170, Escom, Leiden, The Netherlands 1995.
17. W.H. Moore and S. Krimm (1976). Vibrational analysis of peptides, polypeptides and proteins. I. Polyglycine I. *Biopolymers 15*, 2439–2464.
18. M. Mutter, R. Hersperger, K. Gubernator and K. Müller (1989). The construction of new proteins: V. A template-assembled synthetic protein (TASP) containing both a 4helix bundle and a β-barrel-like structure. *Proteins: Struc. Function Genet. 5*, 13–21.
19. M. Narita, S. Honda, H. Umeyama and T. Ogura (1988). Infrared spectroscopic conformational analysis of polystyrene resin-bound human proinsulin C-peptide fragments. β-sheet aggregation of peptide chains during solid phase peptide synthesis. *Bull. Chem. Soc. Jpn. 61*, 1201–1206.

20. T. Miyazawa and E.R. Blout (1961). The infrared spectra of polypeptides in various conformations: Amide I and II bands. *J. Am. Chem. Soc. 83*, 712–719.
21. R.W. Coombs, J.A. Verpoorte and K.B. Easterbrook (1976). Protein conformation in bacterial spinae. *Biopolymers 15*, 2353–2369.
22. T. Akaike, S. Inoue and K. Itoh (1976). Conformation of poly(DL-valine) obtained by the polymerization of the N-carboxy anhydride. *Biopolymers 15*, 1863–1868.
23. K.J. Payne and A. Veis (1988). Fourier transform IR spectroscopy of collagen and gelatin solutions: Deconvolution of the amide I band for conformational studies. *Biopolymers 27*, 1749–1760.
24. P.I. Haris, D. Chapman, R.A. Harrison, K.F. Smith and S.J. Perkins (1990). Conformational transition between native and reactive center cleaved forms of α_1-antitrypsin by Fourier transform infrared spectroscopy and small-angle neutron scattering. *Biochemistry 29*, 1377–1380.
25. M. Narita, Y. Tomotake, S. Isokawa, T. Matsuzawa and T. Miyauchi (1984). Synthesis and properties of resin-bound oligopeptides. 2. Infrared spectroscopic conformational analysis of cross-linked polystyrene resin bound oligoleucines in the swollen state. *Macromolecules 17*, 1903–1906.
26. J.C. Hendrix, K.J. Halverson, J.T. Jarrett and P.T. Lansbury (1990). A novel solvent system for solid-phase synthesis of protected peptides: The disaggregation of resinbound antiparallel β-sheet. *J. Org. Chem. 55*, 4517–4518.
27. M. Narita, H. Umeyama and T. Yoshida (1989). Peptide segment separation by tertiary peptide bonds. Synthesis and conformational analysis of cross-linked polystyrene resin-bound human proinsulin C-peptide fragments. *Bull. Chem. Soc. Jpn. 62*, 3577–3581.
28. B.Due Larsen, D.H. Cristensen, A. Holm, R. Zillmer and O. Faurskov Nielsen (1993). The Merrifield peptide synthesis studied by near-infrared fourier-transform raman spectroscopy. *J. Am. Chem. Soc. 115*, 6247–6253.
29. B. Due Larsen, A. Holm, D.H. Christensen, F. Werner and O. Faurskov Nielsen in: *Innovations and Perspectives in Solid Phase Synthesis*, R. Epton, Ed., p. 363–366, Intercept, Andover 1992.
30. B. Henkel, Versuch der Festphasen-Synthese der HIV 1-Protease-Sequenz 79–99 unter Einsatz von *N*-Methyl-2-pyrrolidinon, 1,3-Dimethyl-tetrahydro-2(1H)-pyrimidinon und des chaotropen Salzes Lithiumchlorid, Diploma thesis, University of Tübingen, 1991.
31. R.C. Sheppard and B.J. Williams (1982). Acid-labile resin linkage agents for use in solid phase peptide synthesis. *Int. J. Peptide Protein Res. 20*, 451–454.

