Pioneer™ Peptide Synthesis System

Monitoring Peptide Synthesis

Introduction

The Pioneer Peptide Synthesis System utilizes a UV detector to measure the absorbance of solutions flowing through the system[1]. This UV data can provide valuable diagnostic and qualitative information about the synthesis process. The Pioneer software provides several choices for feedback control based on the UV data to assist in peptide assembly. Monitoring used in conjunction with other analytical techniques (i.e., HPLC, MS) presents an effective means of measuring the success of a synthesis.

This Technical Bulletin is intended to assist in the utilization of this UV monitoring tool. This includes understanding the factors that can interfere with the interpretation of the detector data as well as methods to reduce these interferences, the use of the feedback monitoring options, and troubleshooting tips.

Detector

Figure 1 illustrates the main components of the Pioneer Peptide Synthesis System. The Pioneer contains a variable speed pump that delivers reagents and solvents through the system.

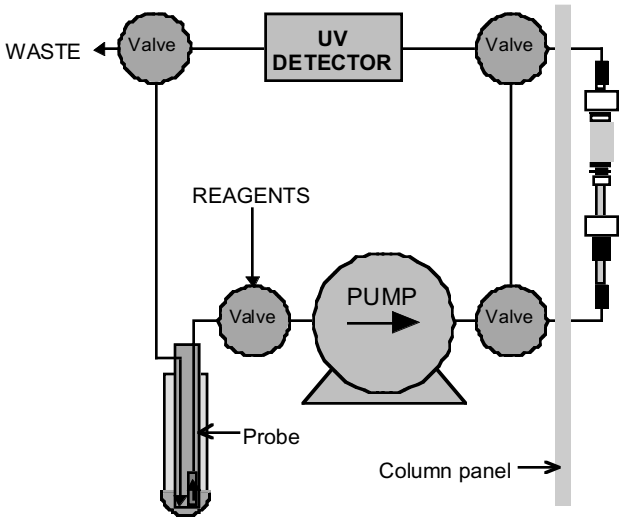

Figure 1. Pioneer system components.

A series of valves directs the fluids or gas through the column to waste during wash or deblock steps, to the tube for dissolution of amino acids, or around a recycle loop (delivery from and to the tube) for coupling of amino acids. The flow can also bypass the column for steps involving priming fluid lines or preactivation and mixing of amino acids and activators.

The post-column UV detector monitors the absorbance as these solutions flow through the system. The detector uses a fixed wavelength (365 nm), single beam optics system with a mercury lamp. The effluent from each column passes through a 0.5 mm quartz capillary flow cell. The signal from the silicon photodiode detector is sent to the CPU in the instrument at 1 second intervals. There are several options for viewing the detector data:

- The detector value, given as analog voltage, on the instrument status screen (from the Main menu, press **Stat** then **Sys**).
- A chart recorder connected to the instrument with the optional chart recorder cables (GEN601381).
- The Instrument window on the Workstation (the detector trace is not active for an MPS synthesis or while the instrument is idle).

A typical detector trace (from a chart recorder or the Workstation) for one synthesis cycle is shown in Figure 2. The appearance of the detector trace may vary depending on the synthesis scale, the cycle, the activation method, and the data sampling rate.

The sequence of steps in a typical synthesis cycle are (the steps correspond to the labels in Figure 2):

A) Remove the *N*-terminal Fmoc protecting group with deblock solution (typically 20% piperidine in DMF). The area of the peak may be proportional to the amount of the Fmoc group removed (see the following section for a detailed discussion of deblock monitoring). Wash the column to remove the deblock solution.

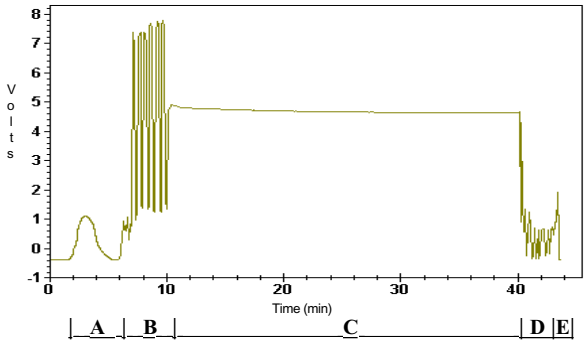

Figure 2. Detector trace.

B) Dissolve and activate the incoming amino acid. The sharp, closely spaced peaks are associated with air flowing through the detector during bubbling of the amino acid solution in the dissolution step. NOTE: This step is performed before step A in a DKP cycle.

C) Couple the activated amino acid to the support by recycling the solution through the column and probe. The oscillating pattern is the activated amino acid solution flowing through the column

Abbreviations: **DIEA**, *N,N*-diisopropylethylamine; **DMA**, *N,N*-dimethylacetamide; **DKP**, diketopiperazine**; DMF**, *N,N*-dimethylformamide; **Fmoc**, fluorenylmethyloxycarbonyl; **HATU**, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; **HBTU**, 2-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; **HOAt**, 1-hydroxy-7-azabenzotriazole; **HOBt**, 1-hydroxybenzotriazole hydrate; **HPLC**, high performance liquid chromatography; **MS**, mass spectrometry; **nm**, nanometer; **NMP**, *N*-methyl-pyrrolidinone; **Pfp**, pentafluorophenyl; **PyAOP**, 7-azabenzotriazol-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; **PyBOP**, benzotriazol-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; **TBTU**, 2-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; **TFFH**, tetramethyl-fluoroformamidinium hexafluorophosphate; **UV**, ultraviolet.

repeatedly. In many cases this will appear as a flat line at the maximum detector reading due to saturation of the detector signal.

D) Wash the probe. The sharp, closely spaced peaks are equated to air flowing through the detector during the probe wash.

E) Wash the support. The excess reagents are washed from the column and the detector returns to a baseline reading.

Deblock Monitoring

The reaction for the removal of the Fmoc group from a peptide-resin using piperidine is shown in Figure 3.

The dibenzofulvene-piperidine adduct (I) is liberated from the support and monitored at 365 nm to give an Fmoc peak on the detector trace (see Figure 2). Ideally, the integrated area of the Fmoc peak is proportional to the quantity of Fmoc removed from the peptide support

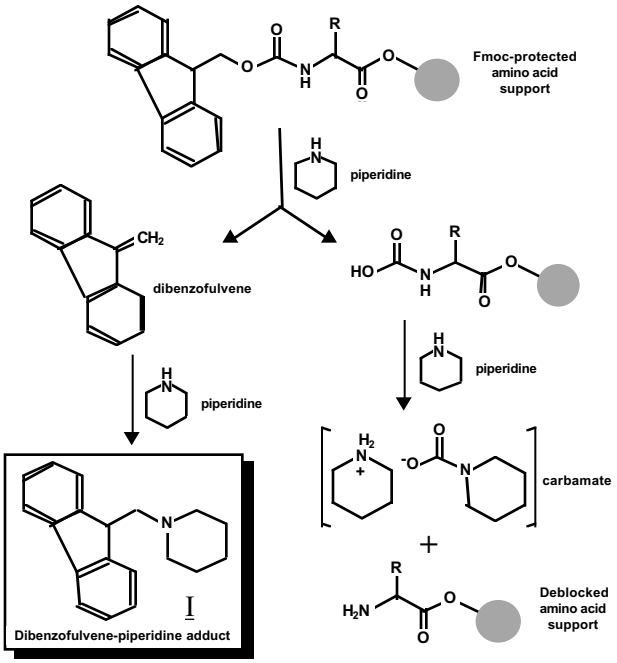

Figure 3. Removal of Fmoc-protecting group.

and the coupling efficiency of the previous amino acid can be calculated. However, there are several factors that reduce the accuracy of this relationship.

- The correlation between the Fmoc concentration and optical density in DMF is not strictly linear. In DMF, the fluorenylmethyl chromophore has a strong fluorescence emission band at 312 nm that can interfere with accurate UV detection[2].
- The extinction coefficient of the dibenzofulvene-piperidine adduct is strongly dependent on the piperidine concentration in the solution[2]. The local concentration of piperidine as the adduct is eluted can vary within a single synthesis. If the deblock reaction is fast, then the adduct is eluted at the leading edge of the piperidine reagent and is mixed with the DMF in the column. In contrast, a slower deblock reaction results in the adduct eluted in a more concentrated solution of piperidine. Also, piperidine is consumed in the reaction by addition to the dibenzofulvene.
  The extinction coefficient of the dibenzofulvene-piperidine adduct was measured in various concentrations of piperidine in DMF. The extinction coefficient was about 2 times greater in a 5% piperidine solution in DMF than in a 20% piperidine solution in DMF. Therefore equal peak areas for successive deblock reactions can only be expected under strictly equivalent experimental conditions.
- Although no other by-products absorb at 365 nm from Fmoc removal, other reagents used during the coupling are detected at this wavelength. In particular, triazole-based coupling reagents (i.e., HOBt, HOAt, HATU, TBTU, HBTU, PyAOP, PyBOP) bind to the solid support and may be eluted during the piperidine wash.

It is difficult to correct for fluorescence effects and extinction coefficient variations in a non-analytical instrument. Although these factors affect the precision of Fmoc peak quantification, the most significant contribution is the interference of the triazole-based reagents.

Monitor Wash

A synthesis performed without a triazole-based activator or additive (i.e., TFFH activation or pre-activated amino acid-OPfp esters without added HOBt) exhibits no monitoring problems as a result of support bound reagents. The same sequence synthesized using triazole-based activators or additives exhibits Fmoc peaks with areas 2 to 20 times higher. The Fmoc peak following a poor coupling can be very large, indicating that the triazole-based reagents also bind to the free amino groups that were not acylated by the incoming Fmoc-protected amino acid.

Figure 4 schematically illustrates the binding phenomenon and the resulting interference in the detection of the Fmoc peak.

- Fmoc-protected amino acid and a triazole-based activator are added to the unprotected peptide support.
- The coupling reaction occurs and the excess reagents are removed with a wash solvent. In one case, a standard DMF wash is used; this does not remove all the bound triazole-based reagent. In the other case, an additional base wash is used which removes the bound triazole-based reagent.
- The Fmoc group is removed with the deblock solution. In addition to removing the Fmoc group, any bound triazole reagent is also removed by the basic solution. The resulting Fmoc peak for the synthesis without the base wash is much higher than the synthesis with the base wash due to the additional absorbance of the triazole reagents.

A variety of methods were employed to eliminate the interference of the bound reagents with the detection of the Fmoc peak (see Table 1).

The bound triazole reagent was removed most effectively by a concentrated base (DIEA) wash after the coupling. No solutions of DIEA in DMF were tested above 20% because more concentrated solutions would separate into two layers. Treatment of the support with neat DIEA (100%) for 1 min was the most efficient wash condition to remove the bound triazole reagent.

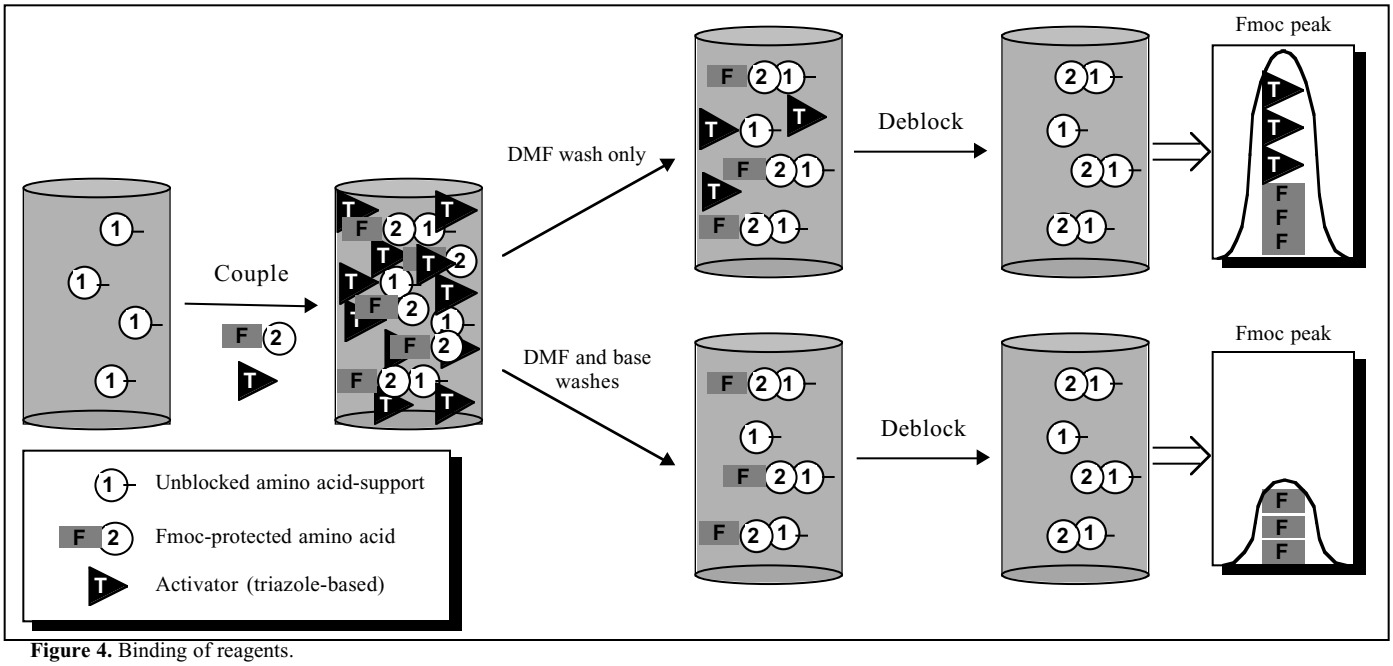

Figure 4. Binding of reagents.

Table 1. Removal of triazole-based reagents.

Experiment	Result
Use a different wavelength to detect the Fmoc peak.	No suitable wavelength. The absorbance profiles of triazole-based reagents and the dibenzofulvene-piperidine adduct overlap significantly.
Use NMP or DMA as a wash solvent after the coupling.	Little improvement in removing bound reagents.
Increase final DMF wash from 4 to 10 min.	Bound reagent not totally removed.
Add a 2 min methanol wash after the coupling.	No improvement in removing bound reagents.
Add a 4 min base wash (5% DIEA in DMF) after the coupling.	Bound reagent not totally removed.
Add a 2 min base wash (20% DIEA in DMF) after the coupling.	Bound reagent not totally removed.
Add a 1 min base wash (100% DIEA) after the coupling.	Bound reagent removed.

The Fmoc group is removed at a slow rate ($t_{1/2}$ ~ 10 h) by DIEA[3]. Thus, a one minute exposure of the Fmoc-protected support to neat DIEA will remove less than 0.2% of the Fmoc group. Premature Fmoc removal is non-problematic in single coupling protocols since the Fmoc group is removed by the deblock reagent following the coupling step. In double coupling protocols, premature Fmoc removal following the first addition of amino acid results in an undesired additional incorporation of this residue in the sequence. To avoid this undesired reaction, the base wash is automatically skipped when the next cycle is a recoupling cycle.

The standard Pioneer Template protocol specifies the Aux 2 bottle position for the DIEA monitor wash. Pure DIEA (GEN903082) should be used for this wash to avoid any undesired side reactions (the 900 mL bottle of distilled DIEA attaches directly to the Aux 2 position on the Pioneer). The monitor wash is only performed if one of the feedback monitoring options is selected for the synthesis (see Feedback Monitoring section for more details).

A DIEA wash is not required to successfully synthesize a pure peptide on the Pioneer. It is useful for more straight-forward interpretation of detector data and is required for feedback monitoring. However, the expense of the added reagent and additional cycle time may not be warranted for the assembly of short or less complex sequences.

Fmoc Histogram

The data for the Fmoc histogram are generated from the detector trace data and are an integration of the peak produced during the deblock steps.

The deblock portion of the Template protocol (Fast cycle) is shown in Figure 5.

	Sub Cycle	Function	Flow (ml/min)	Time (sec)	Description
1	Deblocking	Dblk to waste w/o col	40	8	Flush lines with Deblock
2	Deblocking	Zero detector	2	0	[ZERO] Zero detector
3	Deblocking	Start(1)/Stop(0) data collect	1	0	Start data collection
4	Deblocking	Dblk to waste with col	10	90	[S1] Deblock to column
5	Deblocking	Wash to waste with col	30	50	[S2] Flush column with Wash
6	Deblocking	Start(1)/Stop(0) data collect	0	0	Stop data collection

Figure 5. Deblock protocol steps.

Line 1) Flush lines with Deblock: Primes the fluid lines and fills the detector with deblock solution.

Line 2) Zero detector: Instructs the software to use the current detector value as the baseline for the histogram calculations.

Line 3) Start data collection: Instructs the software to initiate data collection from the detector. The following calculation is performed every second:

histogram value = detector value - baseline value

The individual histogram values are accumulated until the software is instructed to stop data collection at line 6.

Line 4) Deblock to column: Delivers Deblock solution to the column to remove the Fmoc group from the peptide support.

Line 5) Flush column with Wash: Washes the column with DMF to remove the deblock solution.

Line 6) Stop data collection: Instructs the software to stop collecting the histogram data. The sum of the values is displayed as a bar on the histogram.

Figure 6 illustrates a typical histogram for the synthesis (with and without DIEA base wash) of a 15 amino acid containing sequence.

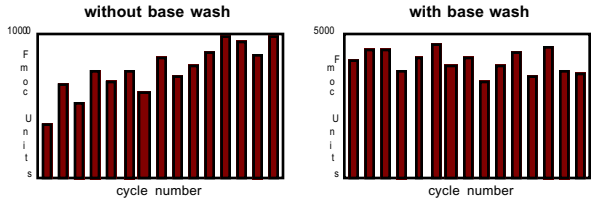

Figure 6. Typical histogram.

The first bar is about the same height both with and without the base wash (note: the Y-axis scales on the histograms are different). Since no coupling has occurred before the deblock, the support has not been exposed to the triazole-based reagent and there is no interference during the deblock. The bars trend upward on the synthesis without the base wash because, in general, as the peptide grows there is more binding of the triazole-based reagents to the support. Additionally, the values for the bars in the synthesis without the base wash are significantly larger than those of the synthesis with the base wash due to the contribution of the triazole-based reagent washout to the peak area.

The synthesis with the base wash shows normal variation in bar heights. During a successful synthesis the bars tend to maintain a constant level or trend slightly downward during the synthesis.

Figure 7 is an example of a histogram of a synthesis with a poor coupling during the second cycle (with and without base wash).

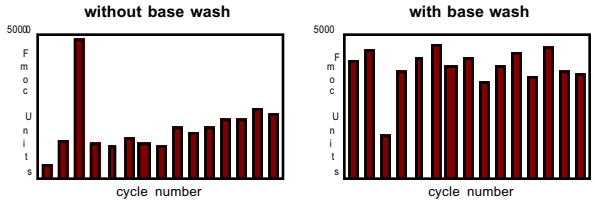

Figure 7. Histogram of poor coupling

There is a large bar at the third cycle since the poor coupling in the second cycle left free amino groups exposed which bind the triazole-based reagents. The same synthesis with the base wash, however, shows a small bar for the third cycle since less amino acid was attached to the solid support and there was no interference from triazole-based reagent binding. Although both histograms indicate that a problem occurred during peptide assembly, the one with the base wash gives a more accurate description.

Feedback Monitoring

Feedback monitoring is a method that modifies reaction times based on the efficiency of the Fmoc removal. A slow Fmoc removal may be attributed to poor accessibility to the growing peptide support and thus the subsequent attachment of the amino acid may be difficult. With slow Fmoc deprotections, an increase in the deblock and/or coupling times may drive the reactions to completion and produce a purer product.

The Pioneer Peptide Synthesis System offers the user several levels of feedback control to assist in the synthesis of the peptide. The following stops describe the deblock monitoring related operations:

1. The deblock reagent is pumped through column to initiate the removal of the Fmoc group from the solid support.
2. The deblock step is continued until either:
   - The step timer has counted down to zero (Auto Extend Steps = No)
     or
   - The step timer has counted down to zero *and* the change in the detector value over a period of time falls below the *slope factor* value or the *maximum deblock time* is reached (Auto Extend Steps = Dblk or Coupl).
3. The subsequent coupling step is continued until either:
   - The end of the coupling step is executed (Auto Extend Steps = No or Dblk)
     or
   - The coupling time in the protocol is multiplied by a factor equal to the ratio of the extended deblock time to the deblock time in the protocol (Auto Extend Steps = Coupl). For example, if the deblock time was extended to twice the time specified in the protocol, then the subsequent coupling time is doubled.
4. The column is washed with DIEA from the Aux 2 bottle to remove any traces of activator bound to the solid support that may interfere in the subsequent deblock step (Auto Extend Steps = Dblk or Coupl).

To access these feedback monitoring options either:

- Change the *Auto extend* options in the Instrument Profile menu.

or

- Change the *Fmoc Monitor* options in the drop-down list boxes in the Properties of the Workstation Instrument window, as described below.

The default *maximum deblock time* is 10 min and can be changed to any value between 0 and 99 min. Increasing the *maximum deblock time* increases the maximum coupling time since the factor used to multiply the protocol coupling time can be greater.

For example, the default maximum deblock time is 10 min and the deblock time in the protocol is 5 min. Therefore, the maximum coupling factor is 10/5 = 2. If the maximum deblock time is increased to 20 min then the maximum coupling factor is 20/5 = 4.

The default *slope factor* is 5 and can be changed to any value between 0 and 99. The *slope factor* is a measure of the stability of the detector signal. A large *slope factor* indicates that the signal can vary more during a set time period whereas a small *slope factor* indicates there can be little variation in detector signal and the deblock is more likely to be extended.

Troubleshooting

Caution should always be exercised in concluding from the histogram that a synthesis has failed. The detector trace should be examined to see if there are any anomalies that can explain unexpected histogram data.

Table 2 describes some features of the detector trace that can be an indication of certain problems in the system.

Table 2. Detector troubleshooting

Observation	Possible Cause
Flat line for entire trace	• Hardware problem (electronics, flow cell cracked) • Bad lamp
Sharp spikes appear during recycle	• Air introduced during recycle due to a valve fault
Many sharp spikes (where air is not supposed to be introduced)	• Air leaking into lines (gas valve or fittings leak) • Reagent(s) depleted
Low signal during recycle	• No amino acid in vial • Clogged probe frit • Wrong activator selected in Notebook
Noisy baseline	• Low lamp energy • Dirty flow cell

The histogram can be also useful in diagnosing problems. Table 3 describes some additional features of the histogram that can be an indication of certain problems.

Table 3. Histogram troubleshooting

Observation	Possible Cause
The first histogram bar is larger or smaller than the other bars.	• The detector was not sufficiently warmed up. If the instrument is idle for 2 h, the detector switches off to conserve the lamp life and does not turn on until a key is pressed on the Pioneer keypad or a synthesis is submitted from the Workstation. If the detector is not stable during the establishment of a baseline value for the histogram calculation, then the value for the first bar will be inaccurate. • The system and/or column was not properly primed and the baseline value was determined with air in the flow cell. • The support does not contain a terminal Fmoc group.
All histogram bars are missing	• Unresponsive detector due to hardware problems (i.e., faulty electronics, dirty or cracked flow cell, bad lamp)
Some bars missing	• Chemistry problem (i.e., poor coupling, depleted or bad reagents). • Baseline value was determined with air in the flow cell.
A large bar for the last cycle when using Fast cycles	• The final cycle uses an extended deblock that may lead to higher total calculated histogram values.

Conclusion

The Pioneer UV detection system provides a useful tool for monitoring the peptide synthesis process. Several factors that may compromise the precision of the detector data were identified. Most significantly, triazole-based reagents bind to the peptide-solid support especially when there are free amino groups. The bound reagents interfere with the detection and quantification of the Fmoc peak. A short DIEA wash can be used after the coupling step to remove the bound reagents and increase the accuracy of the subsequent Fmoc peak measurement.

Although the detector data can provide useful diagnostic and qualitative information, it should not be used exclusively to measure the success of the synthesis or to replace other analytical techniques (i.e., HPLC, MS).

We are always interested in hearing your comments and assisting with any questions you may have. Your comments helps us to continually improve our products. Please direct questions and report any issues to one of our offices listed below.

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[1] For further discussion about UV monitoring on continuous flow peptide synthesizers see Atherton, E. and Sheppard, R.C., Solid Phase Peptide Synthesis- A Practical Approach (1989), IRL Press, pp 107-130.

[2] Dryland, A. and Sheppard, R.C., J. Chem. Soc. Perkin Trans. I, (1986), pp 125-137.

[3] Fields, G.B. and Noble, R.L., Int. J. Peptide Protein Res., 33 (1989), p 21.