User Bulletin No. 29

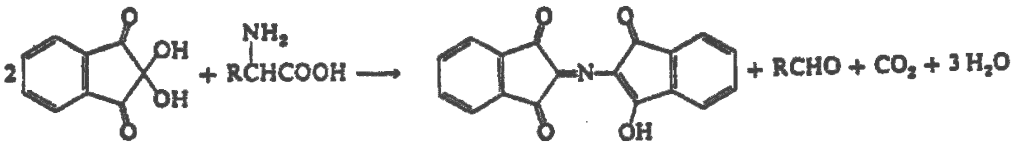
Peptide Synthesizer

July 1989 (updated 09/2002)

SUBJECT: Use of the Ninhydrin Reaction to Monitor Fmoc Solid-Phase Peptide Synthesis

The quantitative ninhydrin monitoring of Boc chain assemblies on Pam resins in Solid-Phase Peptide Synthesis (SPPS) described by Sarin *et al.*[1] can also be used to monitor Fmoc chain assemblies. Two changes are required to assure accurate monitoring with Fmoc-peptide-resins. First, when using the resin sampler on either the Model 430A or the Model 431A Peptide Synthesizers, 2 to 3 drops of glacial acetic acid should be added to the 1-2 mL methanol in the sample test tubes. Second, the test tubes should be heated for 5 minutes instead of 7 minutes. These changes reduce the liability of the Fmoc protecting group to organic bases.

The reaction of amino acids with ninhydrin to give a deep violet blue color was first observed by Ruhemann.[2] This reaction is shown in Scheme 1.



Ninhydrin Ruhemann's Purple

Scheme 1

Applied Biosystems

The ninhydrin reaction was adapted to SPPS by Kaiser *et al*.[3] and later modified by Sarin *et al*. to develop a method for the quantitative determination of the coupling efficiency in SPPS for Boc-peptide Pam resins.

The three reagents used in the ninhydrin monitoring procedure are:

Monitor 1: Phenol in ethanol

Monitor 2: Potassium cyanide in pyridine

Monitor 3: Ninhydrin in ethanol

A completely protected peptide-resin does not produce a blue color with the ninhydrin test. However, the pyridine in Monitor 2 partially removes the Fmoc protection so that a slight blue color results.

In the paper by Sarin *et al*. on monitoring Boc couplings, they state that the maximum color develops within 5 minutes at 100° C, and remains constant for another 10 minutes before the color begins to decay. Sarin *et al*. recommend heating for 10 minutes. The Applied Biosystems protocol[4] recommends heating for 7 minutes. While 7 minutes of heating is still recommended for the Boc-peptide resins, when monitoring Fmoc-peptide resins, the heating time should be reduced to 5 minutes. The following set of experiments will demonstrate the reasons for this change.

Figure 1 compares the results of the ninhydrin monitoring of two protected amino acids: Fmoc-Val and Boc-Val. Clearly, Fmoc-Val is not stable under the ninhydrin monitoring conditions, while Boc-Val is stable under the same conditions. In 7 minutes at 100° C, approximately 0.7% of the Fmoc protecting group is removed from Fmoc-Val.

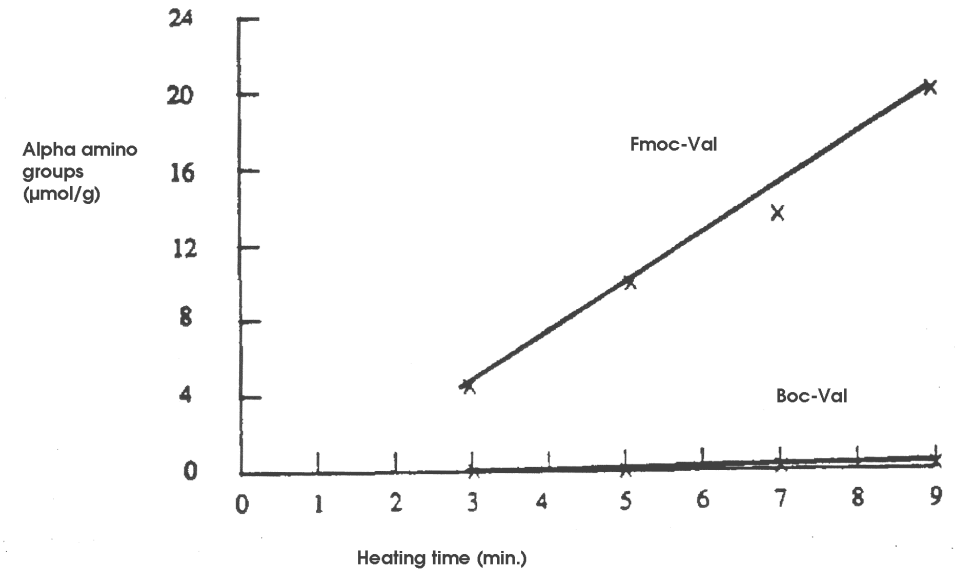


Figure 1. Ninhydrin assay of Fmoc-Val and Boc-Val

Because the Fmoc group is slowly removed under the conditions of the ninhydrin test, we need to determine the optimum heating time for the reaction between the uncoupled peptides and ninhydrin. It must be long enough to ensure that all the uncoupled peptides react, but short enough to limit background due to Fmoc cleavage.

To determine this optimum reaction time, we monitored the ninhydrin reaction with two Fmoc-Leu-Met-resins. One resin was made by coupling the Fmoc-Leu-HOBt ester to Met-resin for only 2 minutes. The coupling efficiency, calculated using the ninhydrin value after 5 minutes of heating, was 96.9%. The second resin was coupled for 30 minutes. The coupling efficiency calculated from the ninhydrin value at 5 minutes is 99.7%. From the graph of results shown in Figure 2, it is evident that the ninhydrin test is complete after 5 minutes.

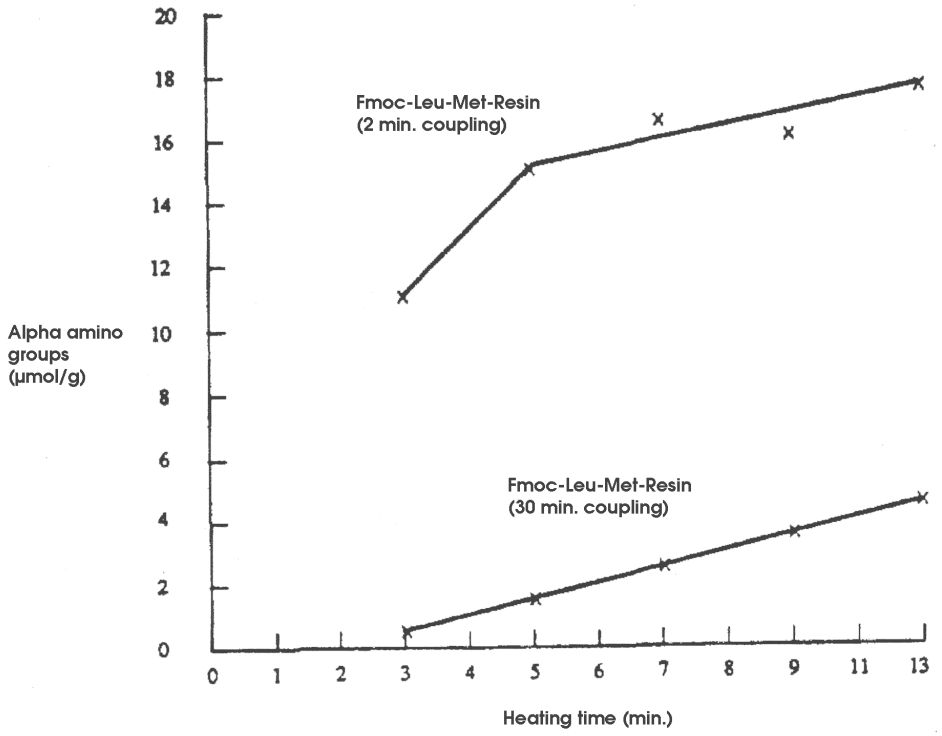


Figure 2. Ninhydrin monitoring of two Fmoc-Leu-Met-resins

We performed the same experiment with Boc-Leu-Met-resin to determine the effect of prolonged exposure to heat on Boc-peptide-residues. The results, shown in Figure 3, indicate that Boc-peptide-resins do not decompose under the conditions of ninhydrin test.

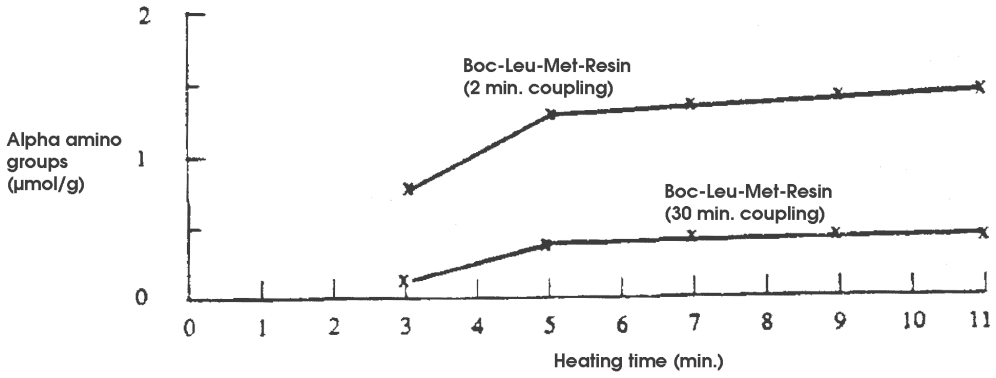


Figure 3. Ninhydrin monitoring of two Boc-Leu-Met-resins

Even if we heat the ninhydrin test with Fmoc-peptide resins for only 5 minutes, we can still expect a background range of 0.5 - 1.0%. As evidence, Table 1 compares the apparent coupling efficiency of Boc vs. Fmoc synthesis of Substance P.

Table 1. Coupling Efficiencies by Ninhydrin Monitoring for the Chain Assembly of Substance P by the Boc and Fmoc methods

	Boc synthesis with MBHA resin		**Fmoc synthesis with Rink amide resin[5]**	
Cycle	Amino Acid	% Coupled	Amino Acid	% Coupled
1	Met(O)	99.8	Met	98.9
2	Leu	99.9	Leu	99.1
3	Gly	99.8	Gly	98.9
4	Phe	99.8	Phe	98.9
5	Phe	99.8	Phe	99.0
6	Gln	99.6	Gln	99.3
7	Gln	99.6	Gln	99.5
8	Pro	99.5	Pro	99.4
9	Lys(Cl-Z)	99.7*	Lys(Boc)	99.3*
10	Pro	99.7	Pro	99.5
11	Arg(Mts)	99.7*	Arg(Mtr)	99.1*
Average coupling		99.7%		99.2%

The chain assembly was done on the Model 431A Peptide Synthesizer using HOBt esters in N-methylpyrrolidone.

* These values do not represent coupling efficiency. Refer to discussion below.

For the Boc synthesis, the average coupling efficiency appears to be 0.5% greater than for Fmoc synthesis (99.7% verses 99.2%). However, examine the values after proline to see the effect of the Fmoc removal. Proline has a secondary amino group and does not react with ninhydrin to form Ruhemann's Purple. Therefore, we expect the values immediately after proline to be 100%—a baseline value for coupling efficiency.

The value immediately after proline in the Boc synthesis is “99.7%.” If we subtract this value from 100%, we define the difference (0.3%) as the background.

The background values after proline in the Fmoc synthesis are “0.7%” and “0.9%.” These values are the results of partial Fmoc deprotection. A background of 0.5% to 1% is typical when monitoring an Fmoc synthesis.

When monitoring Fmoc couplings on the Model 430A and Model 431A Peptide Synthesizer with the resin sampler, we also recommend the addition of 2 to 3 drops of glacial acetic acid to the 1-2 mL methanol in each sample tube.[6] This modification is necessary because the Fmoc group is labile in the presence of trace amounts of methylamine in N-methylpyrrolidone (NMP) and dimethylformamide (DMF).

It has been reported that after 7 days at R.T. in dimethylacetamide (DMA), dimethylformamide (DMF), and NMP, Fmoc-Gly decomposes to the extent of 1%, 5%, and 14%, respectively.[7] A similar study was performed in our labs using Fmoc-Lys(Boc)-resin with these solvents for 20 hours. The results, shown in Table 2, indicate that 35% of the Fmoc group was removed after 20 hours in NMP.

Table 2. Stability of the Fmoc group on Fmoc-Lys(Boc)-resin in various solutions for 20 hours

Resin	Solution	Ninhydrin (µmol/g)	% Deprotected
6.0 mg	1 mL NMP	148	35
4.9 mg	1 mL DMF	14	3
7.0 mg	1 mL DMA	2	0
4.9 mg	1 ml NMP & 50 µL HOAc	2	0
5.3 mg	1 mL 1 M HOBt/NMP	2	0
4.4 mg	1 mL 0.05 M HOBt/NMP	2	0
4.6 mg	100 µL NMP & 200 µL DCM	44	10

However, it is possible to completely eliminate the Fmoc cleavage by adding acetic acid. For this reason, a few drops of acetic acid should be placed in the test tube along with the methanol.

It is important to notice that the Fmoc-Lys(Boc)-resin is stable in NMP when HOBt is present. HOBt is an acedic compound that neutralizes the traces of amines in the NMP. Since all couplings in NMP use amino acids that have been activated with HOBt, there is no Fmoc-group removal during coupling in NMP with HOBt esters.

References

1. Sarin, V.K., Kent, S.B.H., Tam, J.P. & Merrifield, R.B. (1981) *Anal. Biochem.* **117**, 147-157.
2. Ruhemann, S. (1911) *J. Chem. Soc.* **99**, 792, 1306, 1486.
3. Kaiser, E., Colescott, R.L., Bossinger, C.d. & Cook, P.I. (1970) *Anal. Biochem*. **34**, 595.
4. Model 430A User Manual, Version 1.3B (7/1/88) pg. 7-85 to 7-86.
5. Rink, H. (1987) *Tetrahedron Lett*. **28**, 3787-3790.
6. Suggestion made by members of Dr. Geoffrey W. Treagear's Laboratory at the Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Australia.
7. Atherton, E., Bury, C., Sheppard, R.C. & Williams, P.J. (1979) *Tetrahedron Lett*. **32**, 3041-3042.

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