

## A Qualitative Test for Monitoring Coupling Completeness in Solid Phase Peptide Synthesis Using Chloranil \*

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A qualitative test is described which can be carried out in the presence of the reaction mixture. At intervals, a resin sample is removed from the reactor, and the chloranil test is carried out directly. If free amino groups are present, a green to blue color develops on the resin beads in less than five minutes. The test can also be used when the *N*-terminal amino acid residue is proline. By applying the chloranil test, it is demonstrated that the coupling rates depend on the respective amino acid as well as the position in the peptide. The test has been applied to dicyclohexylcarbodiimide couplings and an active ester coupling.

Several qualitative tests for checking the coupling completeness in solid phase peptide synthesis have been reported.<sup>2-5</sup> These tests, however, have to be carried out at a stage of the synthesis, where the coupling mixture is effectively removed from the resin. 2,3,5,6-Tetrachloro-1,4-benzoquinone (chloranil) has been used for qualitative<sup>6</sup> as well as quantitative analyses of amines.<sup>7</sup> It has been shown that chloranil reacts with triethylamine<sup>8</sup> and with diethylamine in the presence of acetaldehyde.<sup>9</sup> In both cases the blue compound 2,3,5-trichloro-6-2'-(*N,N*-diethylaminovinyl)-1,4-benzoquinone is obtained. Furthermore, it is reported<sup>10</sup> that chloranil reacts with ethyl glycinate forming a red product.

### MATERIALS AND METHODS

*Chloranil reagent* consists of a saturated solution of chloranil in toluene. Syntheses were carried out using an automated peptide synthesizer with feed back control.<sup>11</sup> Boc-amino

acids \*\* and DCC were dissolved in dichloromethane where nothing is indicated. The Boc-group was removed by a 1 h treatment with 1 M hydrogen chloride dissolved in a mixture of acetic acid and dichloromethane (9:1, v/v). Boc-amino resins were prepared using the cesium salt method<sup>12</sup> where nothing is indicated. Amino acid analyses were carried out on a Kontron Liquimat 3, and the hydrolyses were carried out as described.<sup>11</sup>

Quantitative acetylation was carried out with acetic acid and DCC and monitored by perchloric acid titrations. If unsatisfactory acetylation was achieved, the acetylation procedure was repeated in the presence of 4-(*N,N*-dimethylamino)pyridine.<sup>13</sup>

The ninhydrin test was carried out as described.<sup>3</sup> HPLC was carried out isocratically on an SP 8000 liquid chromatograph at 40 °C using methanol-water (75/25, v/v) containing 1 % acetic acid as the eluent and monitored by UV at 259 nm, the flow rate being 1 ml/min. A reversed phase column, Nucleosil 10 C-18, was used (length 250 mm, i.d. 4.6 mm).

#### *Procedure for the chloranil test.*

(1) Transfer a sample containing ca. 1 mg of resin to a test tube (50 × 6/7 mm).

(2a) In case of detection of secondary amino groups, 200 μl of acetone is added.

(2b) In case of detection of primary amino-group, 200 μl of acetaldehyde is added.

(3) Chloranil/toluene (50 μl) is added, and the test tube is swirled 5 min at room temperature.

(4) If free amino groups are present, a blue or green color is formed on the resin beads, and the test is positive.

\*\* *Abbreviations:* Boc = *t*-butyloxycarbonyl; Bzl = benzyl-; Ztf = 1-benzylloxycarbonylamido-2,2,2-trifluoroethyl-; ONP = 4-nitrophenylester; Z = benzylloxycarbonyl-; Mbh = 4,4'-dimethoxybenzhydryl; DCC = *N,N*-dicyclohexylcarbodiimid; -S-X1 = 1 % crosslinked polystyrene (Bio Rad); -S-X2 = 2 % crosslinked polystyrene (Bio Rad)

\* Cf. Ref. 1.

Table 1. Comparison of the chloranil test with the ninhydrin test, and the quantitative determinations by amino acid analysis and perchloric acid titration.

| Chloranil test    | Ninhydrin test          |                | Mol Ala/Mol Pro<br>Amino<br>acid analysis | Perchloric<br>acid titration |
|-------------------|-------------------------|----------------|---|------------------------------|
| Strongly positive | Resin: dark brown;      | liquid: blue   | 0.69                                      | 0.71                         |
| Strongly positive | Resin: light brown;     | liquid: yellow | 0.87                                      | 0.89                         |
| Positive          | Resin: light brown;     | liquid: yellow | 0.97                                      | 0.95                         |
| Slightly positive | Resin: light brown;     | liquid: yellow | 0.97                                      | 1.00                         |
| Negative          | Resin: yellow to brown; | liquid: yellow | 1.01                                      | 1.00                         |

## RESULTS

In order to determine the sensitivity in the case of N-terminal proline, a series of peptides were made by coupling Boc-Ala to Pro-Gly-O-S-X2 (0.27 mmol/g), using DCC in less than equivalent amount. Thus peptide resins were prepared with varying amounts of acylated proline residues. After the coupling, samples were taken out, and the chloranil test was compared with the ninhydrin test.<sup>3</sup> Quantitative determinations of the coupling completeness were carried out by amino acid analyses and perchloric acid titrations. The results are shown in Table 1.

Furthermore, the dipeptide Ala-Gly-O-S-X2 was synthesized followed by coupling with Boc-Pro. Boc-Pro, however, was only added in an amount equivalent to 1 % of the alanine residue, but with DCC in excess. When the coupling had proceeded for 15 min, the free amino groups were quantitatively acetylated. The completeness of the acetylation was monitored by perchloric acid titrations, and the chloranil test was negative. After removal of the Boc-group followed by neutralization with triethylamine and 6 washings with dichloromethane, the chloranil test was weak, but clearly positive. The ratio mol Pro/mol Ala was determined by amino acid analysis to be 0.001 and by perchloric acid titration to be 0.02. The two values cannot be considered significantly different.

Similarly, the sensitivity was determined for primary amino groups. Thus, a series of peptides were synthesized by coupling Boc-Pro to Ala-Gly-O-S-X2 with DCC in less than equivalent amount. Also, 1 % Boc-Ala was coupled to Gly-O-S-X2 followed by quantitative acetyla-

tion and removal of the Boc-group. The sensitivity in this case was determined to be in the range of 5–8  $\mu$ mol/g resin when approximately 1 mg of resin was assayed.

A synthesis of the peptide Ala-Val-Pro-Pro-Phe-Gly-O-S-X2 was performed. The couplings were carried out in dichloromethane using Boc-amino acids in 4 times the theoretical amount and DCC in twice the theoretical amount. During the coupling, samples containing approximately 1 mg resin, dichloromethane, Boc-amino acid, DCC, and byproducts, were taken out with a pipette, and the chloranil test was carried out. When the chloranil test was negative, the reactor was drained, and the synthesizer continued the program. After thoroughly washing the resin with dichloromethane and absolute ethanol, resin samples were taken out. A ninhydrin test<sup>3</sup> and a chloranil test were carried out for comparison. Furthermore, the number of residual amino groups was determined by perchloric acid titrations. During the synthesis a slight increase in the titration values after coupling was observed, as if often the case.<sup>14,15</sup> By linear regression the best fit for the slope and the intercept was calculated. The deviation between the calculated and the measured value is used as the estimate for the number of unreacted amino groups. The results obtained are shown in Table 2.

The peptide was cleaved from the resin by hydrogen bromide/trifluoroacetic acid for 2  $\times$  45 min. The following results were obtained by amino acid analysis for the resin bound product. Ala:Val:Pro:Phe:Gly = 0.96:0.94:1.90:1.00:1.02; for the cleaved crude product the corresponding values were 0.99:0.96:1.94:1.00:1.01.

The crude cleaved product was furthermore tested for homogeneity by HPLC. The major

Table 2. Results obtained during the synthesis of Ala-Val-Pro-Pro-Phe-Gly-O-S-X2.<sup>a</sup>  
a. Analytical results obtained during the coupling procedure.

| Ala -<br>C                    | Val -<br>C                    | Pro -<br>C                    | Pro -<br>C                    | Phe - Gly-O-S-X2<br>C         |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| + (1 min)                     | + (1 min)                     | + (1 min)                     | + (1 min)                     | [-] (1 min)                   |
| - (6 min)                     | + (6 min)                     | + (6 min)                     | [+] (6 min)                   | - (6 min)                     |
| Total coupling<br>time 12 min | + (12 min)                    | + (12 min)                    | [-] (12 min)                  | Total coupling<br>time 12 min |
|                               | [+] (18 min)                  | [+] (18 min)                  | - (16 min)                    |                               |
|                               | - (24 min)                    | - (24 min)                    | Total coupling<br>time 22 min |                               |
|                               | Total coupling<br>time 30 min | Total coupling<br>time 30 min |                               |                               |

b. Analytical results obtained after the coupling procedure.

| C N T                     | C N T                     | C N T                     | C N T                     | C N T                     |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| -- -1.3 $\mu\text{mol/g}$ | -- -4.7 $\mu\text{mol/g}$ | -- +6.4 $\mu\text{mol/g}$ | -- +0.8 $\mu\text{mol/g}$ | -- +4.4 $\mu\text{mol/g}$ |

<sup>a</sup> C: Chloranil test, N: ninhydrin test, +: positive test, [+]: slightly positive test, -: negative test, [-]: questionable, T: number of free amino groups after the coupling determined by perchloric acid titration, standard deviation 2.3  $\mu\text{mol/g}$ . The number of minutes in the parentheses shows the coupling time, when samples for the chloranil test were withdrawn from the reactor.

peak had a retention time of 211 s and an area percent of 89 %, a minor peak with retention time 152 s having a shoulder with retention time of 165 s was also present.

In order to elucidate a more general application of the method, synthesis of the following resin bound peptides Tyr(Bzl)-Asn-Pro-His(Ztf)-Gly-O-S-X2, Lys(Z)-Sar-Gln(Mbh)-Leu-O-S-X2, and Asp(Bzl)-Ile-Ser(Bzl)-O-S-X1 was carried out. The couplings were monitored by the chloranil test as described above. The coupling completeness was determined by the ninhydrin test. Boc-amino acids were added in four times the theoretical amount. DCC was used in twice the theoretical amount as well as 4 times the theoretical amount. Similarly, couplings with Boc-Cys(Ztf) and Boc-Arg(NO<sub>2</sub>) have been performed.

The following resins were all negative in the chloranil test, Boc-Trp(CHO)-O-S-X2, Boc-Glu(Bzl)-O-S-X2, and Boc-Thr(Bzl)-O-S-X2. Furthermore, Boc-Met-NH<sub>2</sub> did not develop a colored product when submitted to the chloranil test.

Boc-His(Ztf) was coupled in the presence of hydroxybenzotriazole. It turned out that chloranil gives a faint violet to rose color with histidin containing peptides, thus blurring the chloranil test for coupling completeness, though not precluding the test in this case. The aspar-

agine residue was incorporated using an active ester coupling. Boc-Asn-ONP was dissolved in DMF-CH<sub>2</sub>Cl<sub>2</sub> (1:10 v/v). After a coupling time of 24 h, the chloranil test was still positive, thus quantitative acetylation had been carried out. By perchloric acid titrations it was indicated that 9  $\mu\text{mol/g}$  or 4 % of the amino groups were acetylated.

During the coupling with Boc-Ile in the synthesis of Asp(Bzl)-Ile-Ser(Bzl)-O-S-X1 it was observed, when the chloranil test was carried out, that the bulk amount of the resin beads were uncolored. Individual beads, however, were green colored, indicating that the amino groups present on the different resin beads are dissimilarly accessible.

Boc-amino resins prepared from the triethylammonium salt of the Boc-amino acid-chloromethylated resin with ethanol as solvent<sup>16</sup> gave dark colored resins when submitted to the chloranil test. This is probably due to the presence of free amino groups on the resin. A Boc-Phe-resin prepared from a quarternary ammonium salt-chloromethylated resin with dioxane as solvent<sup>17</sup> did not give coloration in the chloranil test.

## CONCLUSIONS AND DISCUSSION

From the results obtained it can be seen that the sensitivity for detection of *N*-terminal

proline is in the range of 2–5  $\mu\text{mol/g}$  resin and for primary amino groups 5–8  $\mu\text{mol/g}$  when approximately 1 mg of peptide resin is assayed.

Furthermore, it is demonstrated that the required coupling time depends on the amino acid as well as the position in the peptide.

By comparing the results obtained by perchloric acid titrations and the chloranil test, it is seen that the sensitivity of the chloranil test carried out in the presence of the coupling mixture is comparable to the sensitivity where the reaction mixture was effectively removed from the resin.

Several peptide syntheses have been carried out monitored by the chloranil test indicating that the method is generally applicable. However, when histidine is present, the test result may be blurred.

In a few cases, discrepancy between the chloranil test and the ninhydrin test was observed. Thus, after coupling with Boc-Arg ( $\text{NO}_2$ ), Boc-Asp(Bzl), Boc-Sar, and Boc-Gln(Mbh), the chloranil test was negative, while the ninhydrin test was positive or weakly positive. By submitting the corresponding Boc-amino acids to the ninhydrin test, however, the liquid became blue or greenish. It has been noted<sup>4,5</sup> that the drastic conditions required for the ninhydrin test may give rise to misleading results. In the active ester coupling of Boc-Asn-ONP mentioned above, the ninhydrin test was negative after 24 h of coupling, while the chloranil test was clearly positive.

A coupling time of 2 or 4 h and even longer is normally used in solid phase peptide synthesis. By monitoring the coupling reaction with the chloranil test, it is possible to determine when the acylation is complete, so that the synthetic procedure can be continued. The time for the synthesis can thus be reduced.

Preliminary experiments have most likely shown that it is possible to make a fast semi-quantitative determination of the amount of activated Boc-amino acid derivatives present during a coupling. It should thus be possible to monitor the rate for the inactivation of an activated amino acid derivative in a DCC-coupling. The inactivation is caused by formation of an *N*-acylurea derivative. A known amount of a primary or secondary amine, *i.e.* pyrrolidine, is added to a small sample fol-

lowed by a chloranil test. If all the amine is acylated, no blue or green color develops. On the contrary, if the amine is not quantitatively acylated, a blue or green color will develop showing that the amount of activated derivative is reduced.

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