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# Color Test for Selective Detection of Secondary Amines on Resin and in Solution

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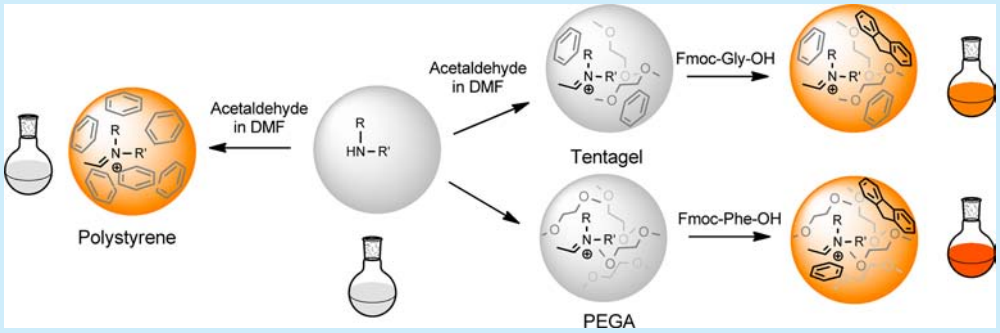
Supporting Information




**ABSTRACT:** Resins for solid-phase synthesis give orange to red-brown resin beads selectively when secondary amines are present on the resin when treated with a solution of acetaldehyde and an Fmoc-amino acid in NMP. The method shows good specificity and gives colorless beads when exposed to a variety of other functional groups. Furthermore, the acetaldehyde/Fmoc amino acid method can be used as a selective colorimetric test for secondary amines in solution.

Amines are important functional groups in synthesis, both in solution and on solid phase. In solid-phase synthesis, direct spectroscopic "on-resin" analysis of amines is limited and a variety of colorimetric methods have been developed to monitor the presence and transformation of amines during the course of the solid-phase synthesis.[1] For the visualization of primary amines, the ninhydrin test developed by Kaiser has found extensive use both in solid-phase peptide synthesis (SPPS) and in solid-phase organic chemistry (SPOC).[2] Later, reported methods for the colorimetric visualization of amines on solid phase involve the use of dyes such as 1-methyl-2-(4′-nitrophenyl)-imidazol[1,2-*a*]pyrimidinium perchlorate (DESC) which form orange to red colored beads in the presence of primary and secondary amines as well as thiols.[3] Other methods involve the use of trinitrobenzenesulfonic acid (TNBSA),[4] substituted naphthoquinones, and 4-*N*,*N*′-dimethylaminoazobenzene-4-isothiocyanate (DABITC) which detect both primary and secondary amines.[5,6] Also nondestructive "reversible" colorimetric monitoring methods for primary, secondary amines and thiols have been reported.[7] The isatin test is the hitherto only reported method for the selective detection of secondary amino acid residues (proline) on solid phase.[8] A single paper by Feigl and Anger[9] reported that acetaldehyde combined with sodium nitroprusside could selectively detect secondary amines in solution, and the method was used for quantitative analysis of secondary amines as well. Here, the authors reported that the color of the solution faded out after a few minutes.[10] A method which has been extensively applied for the visualization of both primary and secondary amines on solid phase is the chloranil method which is performed by adding two drops of a 2% acetaldehyde in DMF followed by the addition of two drops of 2% chloranil in DMF. Applying DMF as solvent instead of toluene increases the sensitivity of the chloranil test.[11,12] As our research involves the formation of various secondary amine intermediates on solid phase in the synthesis of peptides by the backbone amide linker strategy[13,14] we wanted to implement the chloranil test as a versatile test to visualize the presence and reaction of secondary amines on the resin. Whereas the chloranil test has no selectivity between primary and secondary amines, we surprisingly found that, upon adding only one of the test solutions, 2% acetaldehyde in DMF, onto a secondary amine substrate on polystyrene resin (Table 1, entry 3), the resin changed color within 3−5 min at room temperature resulting in orange to dark red resin beads. Upon exposing a primary amine resin to the acetaldehyde solution, no color change of the beads was observed (Table 1, entry 7). To investigate whether this was a general phenomenon or only specific for *N*-methylamine substrates we applied the test to other secondary amine substrates and found a similar color change. In some cases, e.g. with long chain

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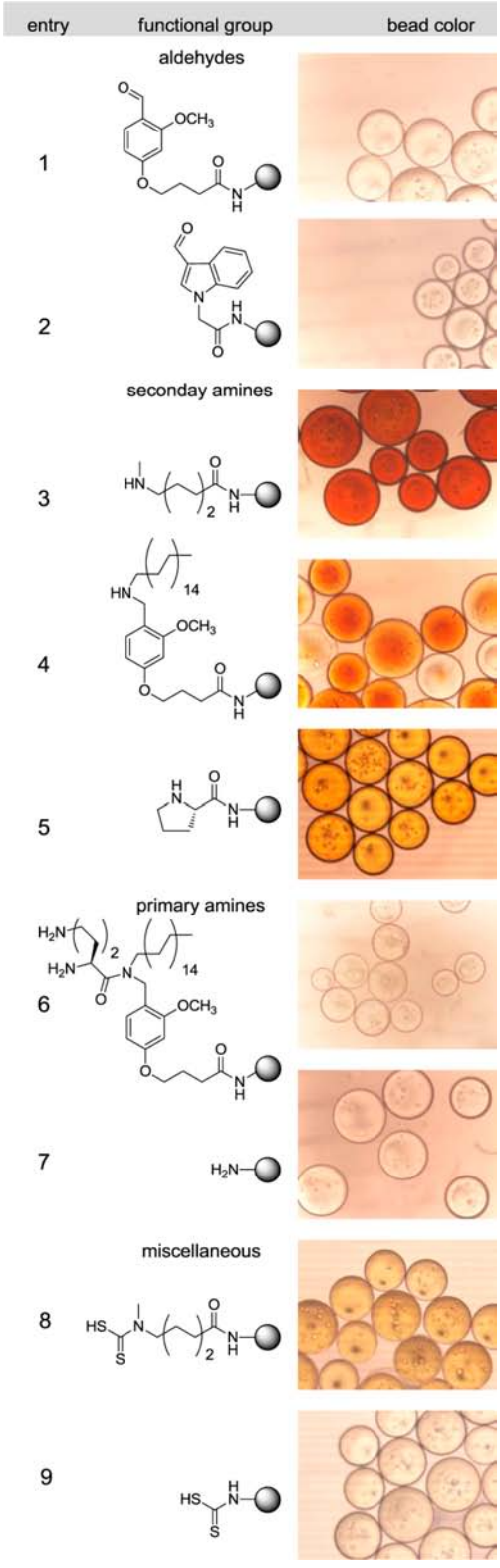
**Table 1. Micrographs of the Acetaldehyde Test Selectivity toward Different Functional Groups on Polystyrene Beads**[15]




alkyl amine substrates, the color change was somewhat slower and needed 5 min for proper visualization (Table 1, entry 4).

To evaluate the applicability of the method to differentiate between primary and secondary amino acids we conjugated Fmoc-protected lysine,[15,16] proline (Table 1, entry 5), and alanine[15,16] to aminomethylated polystyrene. Performing the acetaldehyde test on the Fmoc-protected amino acids resulted in colorless beads,[16] whereas the unprotected amino acids, the proline secondary amine, gave a positive reaction displaying orange colored beads. Here, the acetaldehyde test proved useful as a complementary test to the Kaiser test for primary amines (Figure 1).

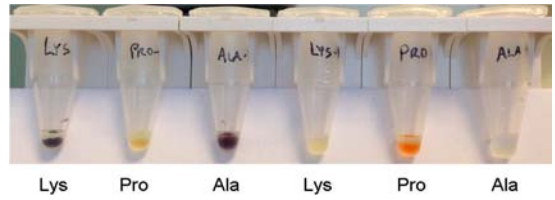



**Figure 1.** Analysis of primary and secondary amino acids on polystyrene resin. (Left) Ninhydrin test detecting primary amines on lysine and alanine (blue colored beads), but giving weakly yellow beads with proline. (Right) "Acetaldehyde test" detecting the secondary amine on proline (orange colored beads) but giving colorless beads with primary amino acids.

As a reference, we investigated several solvents for the solution of acetaldehyde with NMP and THF, which gave similar results.[17] Also different batches of acetaldehyde and polystyrene resins gave the same effect.

The specificity of the test was investigated on a variety of substrates synthesized on solid phase (Figure 1). The type of polystyrene resin resulted in minor differences in the color, where the 1% DVB polystyrene resin gave orange to deep red colors and the rigid macroporous polystyrene gave a more brown-red coloration of the beads. We found that it was necessary to quench the reaction by washing the resin beads with NMP; otherwise colorless beads gradually turned orange upon standing overnight. When the color reaction was quenched both positive and negative test samples could be stored at room temperature without alteration of their color. In comparison, colored resin beads from a positive Kaiser test gradually lost their color upon standing. Upon derivatization of the primary amine with carbon disulfide to form the corresponding dithiocarbamate, the acetaldehyde test showed a negative result (Table 1, entry 9).

However, upon formation of the dithiocarbamate of the secondary amine (Table 1, entry 8) the acetaldehyde test gave a weak positive readout. This is an indication that it is not the nucleophilic dithiocarbamate that gives a positive reaction but merely its decomposition to the (secondary) amine and carbon disulfide.

Acylation of secondary amines to form substituted amides gave colorless beads in the test (Table 1, entry 6). After having observed that the formation of Schiff base adducts between acetaldehyde and secondary amines does not give rise to colored products in solution we investigated the utility of the test on other types of resins by derivatizing PEGA and Tentagel (TG) resins with proline. On these resins we did not observe any color change specific to the presence of secondary amines.[18] The resins changed color upon heating for a few minutes; however, this coloration was unspecific and presumably due to decomposition of the resin. This indicates that this color reaction is dependent on specific properties of the polystyrene resin and with resins such as TG and PEGA the color reaction does not occur. Upon carrying out the acetaldehyde test on primary amino acid polystyrene resins we observed an orange-red colored supernatant. We assumed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the coloration arose from the combination of acetaldehyde and traces of piperidine and dibenzofulvene (DBF) from the preceding Fluorenyloxycarbonyl (Fmoc) deprotection step in the synthesis. As the Fmoc amino acid will rapidly liberate DBF selectively with secondary amines, a small amount of Fmoc-amino acid (Fmoc-Gly-OH) was added to the acetaldehyde solution prior to the retesting of the secondary amino acid proline on Tentagel.[19] Indeed then the proline-Tentagel resin became rapidly colored. In comparison, Tentagel with a primary amine gave no color reaction, indicating the preserved selectivity of the test (Figure 2).

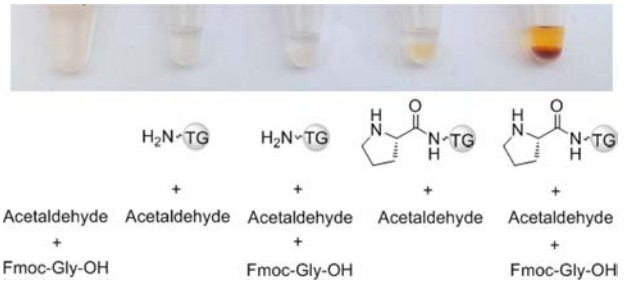


**Figure 2.** Analysis of primary and secondary amino acids on Tentagel, effect of adding Fmoc-Glycine to the acetaldehyde solution.

The yellow colored supernatant formed during the color test could be removed by washing with NMP, while the beads remained orange-red colored. Proline on PEGA resin which has a very low content of aromatic residues also gave a color reaction with the acetaldehyde/Fmoc-Gly-OH solution. Here, it was investigated whether the addition of aromatic amino acids such as Fmoc-phenylalanine or Fmoc-tryptophane would give a stronger "readout". The acetaldehyde/Fmoc-phenyl alanine mixture gave a more rapid coloration (approximately 1 min) of the PEGA resin compared to the acetaldehyde/Fmoc-glycine mixture (3 min) and a somewhat more deep red-brown coloration of the resin beads. The sensitivity of the acetaldehyde/Fmoc-phenyl alanine method was tested on polystyrene and tentagel resins similar to the methods described by Claerhout et al.[3] and Yang et al.[7] Here a visible color change could be observed in loadings down to ca. 3−6 $\mu$mol/g where Tentagel gave visible coloration within 5 min compared to the reference resin; polystyrene resin required 10 min for a clear visual readout.[16]

The utility of the acetaldehyde/Fmoc-amino acid method for colorimetric analysis of secondary amines in solution was also investigated. We used *N*-benzylmethylamine (**A**) as a model system. Mixing of acetaldehyde and **A** in NMP or DMF gave no coloration; however, upon the use of acetaldehyde/Fmoc amino acid mixtures the solution became colored within 1−3 min. As a reference, benzyl amine (**B**) was exposed to the same treatment and here no color development was observed (Figure 3).

We tested the sensitivity of the method for the test of secondary amines in solution and found that the coloration could be visually observed in concentrations down to 0.3−0.6 mM.[16] At low concentrations (0.25 mM) no increased sensitivity was observed upon using 10% acetaldehyde solution or upon increasing the concentration of the Fmoc-amino acid (data not shown). Although we are not certain of the mechanism behind the color reaction, it is evident from our solid-phase experiments that polystyrene exposed to the

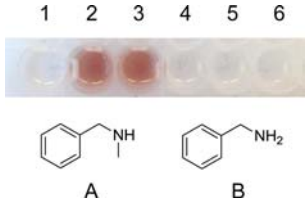


**Figure 3.** Color test carried out in solution (amine concentration 500 mM). (1) **A** and acetaldehyde; (2) **A**, acetaldehyde, and Fmoc-Gly-OH; (3) **A**, acetaldehyde, and Fmoc-Phe-OH; (4) **B** and acetaldehyde; (5) **B**, acetaldehyde, and Fmoc-Gly-OH; (6) **B**, acetaldehyde, and Fmoc-Phe-OH.

iminium/enamine adduct from acetaldehyde and the secondary amine gives the coloration, whereas in more "polystyrene deficient" resins such as Tentagel and PEGA, or in solution, this color reaction is not observed. Furthermore, small amounts of DBF from the Fmoc-amino acid seem to play a key role in the formation of colored adducts with the iminium/enamine, both on resin and in solution. It is known that DBF undergoes spontaneous anionic polymerization when exposed to nucleophiles such as e.g. alkoxides,[20−23] and enamines can perform Michael type addition to electrophilic alkenes.[24] Therefore, it may be proposed that the enamine formed between the secondary amine and acetaldehyde reacts with small amounts of DBF released *in situ* from the Fmoc-amino acid by the secondary amine and the resulting adduct reacts further with DBF to undergo polymerization (Figure 4).

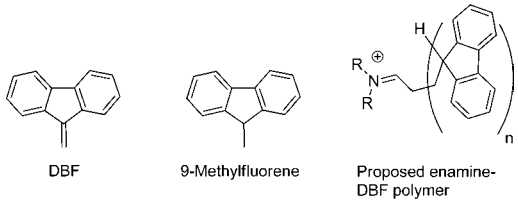


**Figure 4.** Structures of DBF, 9-methylfluorene, and the proposed structure for iminium-Poly-DBF.

To investigate the assumption of enamine induced polymerization of DBF, we set up an experiment where 9-methylfluorene was added to the acetaldehyde test solution instead of the Fmoc-amino acid. Being saturated at the 9 position, 9-methylfluorene would not be prone to Michael addition from the enamine (Figure 4). Whereas the reaction between amine **A** and acetaldehyde in the presence of Fmoc-Phe-OH resulted in coloring of the solution, no color reaction was observed in the presence of acetaldehyde and 9-methylfluorene under similar conditions.[16] This could imply that the role of dibenzofulvene as a Michael acceptor is important for the formation of colored dyes. The MS and IR spectra obtained from the isolated colored dye precipitates from amine **A** and acetaldehyde with Fmoc-Gly-OH or Fmoc-Phe-OH showed similar peaks. This indicates that the same dye is formed regardless of the Fmoc-amino acid used. MS analysis showed molecular ions which could be assigned to several oligomers of the poly-DBF-iminium structure shown in Figure 4.[16] Also the IR of the solid dyes showed a band at 1660 $cm^{-1}$ relating to the iminium group.[16,25] In divinylbenzene (DVB) cross-linked polystyrene resins residual alkenes from DVB may be present from incomplete polymerization.[26] These reactive alkenes may react with the enamine formed from the secondary amine so


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that no additional Fmoc-amino acid is needed to induce coloring in this case. Furthermore, an increased amount of aromatic residues in the system also seems to play a role in the intensity of the color.

In summary, the acetaldehyde test is a simple and fast test to detect secondary amines on polystyrene resins with high specificity. The sensitivity of the method can be enhanced by the addition of Fmoc-amino acids, which makes the method generic for colorimetric analysis of secondary amines on a variety of resin types as well as for visualizing secondary amines in solution. Finally, the 2% acetaldehyde and the 2% acetaldehyde/Fmoc amino acid solutions gave reproducible results after several weeks of storage in the fridge.[27,28]

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental details on synthesis and analysis on selected resin bound substrates; experimental details and results on the following: Measurements of the test sensitivity on solid-phase and in solution; the use of 9-methylfluorene versus Fmoc amino acid; experimental procedures on the preparation and isolation of colored dye precipitates together with MS and IR spectra on isolated colored dye precipitates from exposure of *N*-benzylmethyl amine to the acetaldehyde/Fmoc-amino acid test solution. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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(15) The resin functional groups were verified by infrared spectroscopy; selected spectra can be found in the Supporting Information.
(16) See Supporting Information.
(17) We found that NMP was preferred to DMF as solvent. DMF decomposes upon standing under formation of small amounts of dimethylamine which consumes the acetaldehyde and quenches the color reaction.
(18) Proline on Tentagel gave a weak positive reaction (yellow beads) upon standing for 10 min.
(19) For the seminal paper on the fluorenyloxycarbonyl (Fmoc) group, see: Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404−3409.
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(27) Color test for secondary amines on resin by acetaldehyde/Fmoc amino acid: The test solution was prepared by dissolving Fmoc-Gly-OH (2 mg, 6.7 mmol) or Fmoc-Phe-OH (3 mg, 6.7 mmol) in 2% acetaldehyde in NMP (1 mL). Then a few resin beads were placed in an Eppendorf tube, two drops of the acetaldehyde/Fmoc-amino acid solution were added, and the resin suspension was allowed to stand for 3−5 min at room temperature. Upon the presence of secondary amines the resin beads turned red. The resin beads were washed five times with NMP to quench the color reaction.
(28) Color test for secondary amines in solution: To an amine containing a sample in NMP (200 μL), a test solution (40 μL) comprising Fmoc-Phe-OH (2.6 mg, 6.7 μmol) dissolved in 2% acetaldehyde in NMP (1 mL) was added. The solution incubated for 5 min; orange to red-brown coloring of the solution indicates the presence of a secondary amine in the mixture.