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A sensitive and practical colorimetric test for polymer-supported hydroxyl and thiol groups

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ABSTRACT

A new sensitive and practical colorimetric test for solid-supported hydroxyl and thiol groups is described. The assay is based on the direct labeling with low amounts of commercially available NF31 in the presence of DMAP at room temperature, resulting in easily detectable red beads.

In recent decades, solid-phase chemistry has established itself as a useful tool within fields concerning repetitive oligomer synthesis (peptides, oligonucleotides, etc.) and parallel synthesis of smaller-sized organic molecules, especially due to its amenability to (automated) library construction. However, despite its usefulness, its main disadvantages are the difficulties associated with on-bead product characterization and 'real time' monitoring of on-resin synthetic reactions.[1] Although a number of analytical techniques (NMR, IR, etc.) have been devised to address these shortcomings, a truly routine 'TLC-equivalent' monitoring tool without the need for specialized hardware is difficult to be found and currently it is only approximated by colorimetric tests. These tests are based on the selective staining of reactive polymer-bound functional groups of interest, after which the resulting level of bead-coloration provides a quick glance at the identity of the product or the stage of the reaction at hand. Although initially color tests were especially developed for resin-bound amines (for the monitoring of peptide synthesis), alternative staining strategies have been reported for a number of other reactive functional groups.[2,3]

The detection of resin-bound hydroxyl functions is of special interest. Generally, practical methods for the reliable and sensitive detection of alcohols would be of great utility in solid-phase chemical reactions involving the appearance and disappearance of this functional group, ranging from functional group transformations in small-molecule syntheses to monitoring coupling efficiency in the repetitive construction of oligomers like depsides, depsipeptides, or oligosaccharides and their analogues.[4–6] Moreover, it would allow the general monitoring of the attachment of a first synthetic building block onto popular hydroxyl-derivatized solid supports.

In the past decade, a few groups have reported on the detection of solid-supported hydroxyl groups. Quantitative tests have been developed by the groups of Yan (UV quantification of excess 9-anthroylnitrile reagent in supernatant),[7] Taddei (UV quantification of dimethoxytrityl cation after an on-bead protection/deprotection sequence),[8] and Stien (NMR analysis of excess nitrobenzoylchloride reagent in supernatant).[9] Although these strategies offer the possibility for quantitation, they do not meet the requirements of a fast TLC-like routine test. Next to the above-mentioned quantitative methods, a number of qualitative tests have appeared in the literature. While groups of Yan,[7] Taddei,[8] and Routledge[10] used 9-anthroylnitrile, cyanuric chloride/fluorescein, and *N*-methylisatoic anhydride, respectively, for fluorescence-based bead staining, visual color tests were reported by Riguera (TsCl/nitrobenzylpyridine/piperidine sequence),[11] Taddei (cyanuric chloride/Alizarin sequence),[8] Brown (diphenylsilyldichloride/Methyl Red sequence),[12] Ito (cyanuric chloride-linked Disperse Red)[13] and Komba (Methyl Red/DIC/DMAP).[14] However, in our opinion, these tests suffer from several shortcomings, ranging from impractical multistep sequences (Riguera, Taddei, Brown), over the use of elevated temperatures (Riguera, Taddei, Routledge) or copious amounts of reagents (Brown, Komba), to (in our hands) irreproducible results (Taddei, Brown) and the rather moderate (>16 μmol g^{-1}: Yan, Riguera, Taddei, Brown, Komba) or unreported (Ito) sensitivity.

In our laboratory, we have developed and applied an alternative highly sensitive and practically straightforward colorimetric assay

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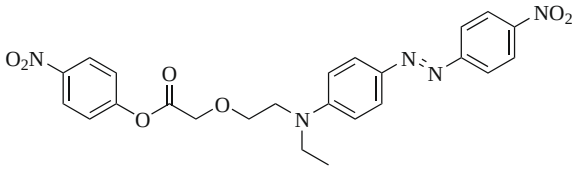

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Figure 1. Structure of NF31 (**1**).

for the direct visualization of resin-bound nucleophiles. In contrast to earlier reported tests, the procedure we wish to present is based on the direct, one-step labeling of the resin-bound functional group using small amounts of commercially available reagents under convenient conditions.

The test is adopted from the known staining method of amines using *p*-nitrophenyl ester **1** (NF31, Fig. 1).[15] This coloring reagent caught our attention because it consists of a sterically accessible activated carboxylic acid ester which has been claimed to react with sterically hindered and weakly-nucleophilic amines using only small quantities of dye.[16] Additional merits are its commercial availability[17] and its good stability, even in stock solution.

However, upon treatment of hydroxylated Wang resin using the originally reported conditions for amine staining (70 °C, 10 min),[18] the results were disappointing, as the treatment only led to nearly colorless beads. Nevertheless, upon addition of an amount of DMAP in an attempt to increase the reactivity of the acylating agent,[19] a very strong color developed, even at room temperature (Fig. 2).

The test is very convenient: a small amount of both a stock solution of NF31 and DMAP in acetonitrile (both stable for months at −20 °C) are added to a small sample of resin, and the mixture is allowed to stand at room temperature for 10 min. The excess of unreacted coloring agent is washed away, leaving the beads available for inspection.[20]

Using this protocol, a number of functionalized resins were tested. While good nucleophiles like the primary amine in aminomethyl polystyrene obviously give an equally intense red color as compared to the above-mentioned Wang resin, Merrifield, 2-chlorotritylchloride, and carboxy-substituted resins remain colorless. Alternative types of hydroxylated supports also react smoothly and show a bright coloration (Fig. 3).

To further evaluate the reactivity of the NF31/DMAP reagent combination and establish the scope and the sensitivity of the colorimetric assay against different functional groups, we investigated a number of resin-bound alcohols and thiols in more detail. It is worthwhile considering also the latter functionality because (besides other more specific applications) the detection of thiol groups on solid supports can be especially of use when cysteine side chains have to be monitored, for instance in disulfide bond formation. A quantitative analysis for resin-bound thiols has been reported (by UV analysis after treatment with Ellman's reagent[21]), but although the possibility for qualitative sulfhydryl determination is mentioned, no detailed information is given.[22] We thus have applied the NF31/DMAP protocol on resins containing primary and secondary alcohols, phenols, and primary thiols. To this end we gratefully exploited the ready availability of suitably protected amino acids: commercially available Fmoc-Ser(O*t*-Bu)-OH and Fmoc-Thr(O*t*Bu)-OH were used for the detection of primary and secondary alcohols, respectively, while Fmoc-Tyr(O*t*-Bu)-OH and Fmoc-Cys(Mmt)-OH were most useful in the determination of, respectively, resin-bound phenols and thiols. The general strategy involves (1) the coupling of a subequivalent amount of protected amino acid to aminomethyl polystyrene, followed by (2) capping by complete acetylation of the remaining amino groups (evidenced by a negative NF31/DMAP test), (3) Fmoc-quantitation to determine the absolute amount of resin-bound amino acid, (4) deprotection of the amino acid side chain to expose the alcohol,

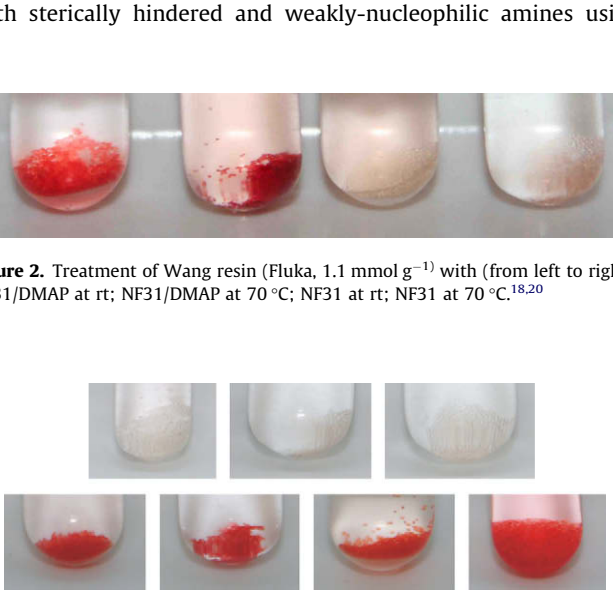
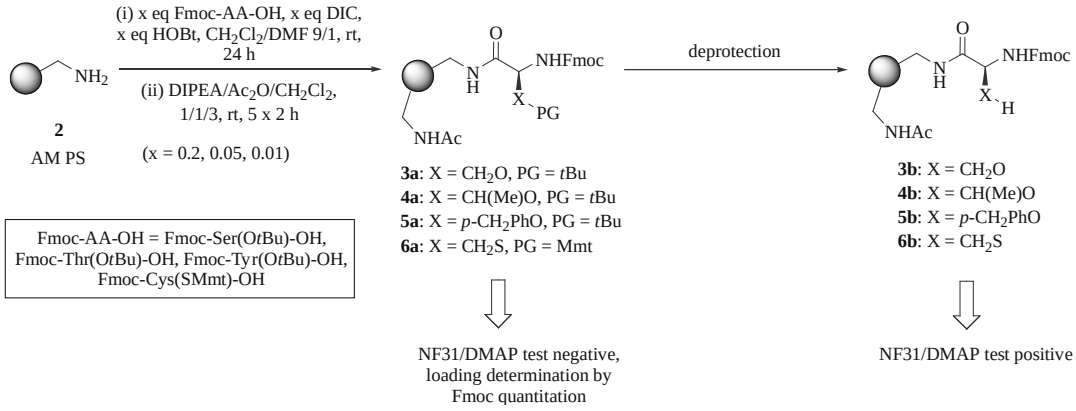


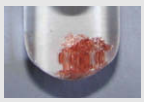
Figure 2. Treatment of Wang resin (Fluka, 1.1 mmol g^{-1}) with (from left to right): NF31/DMAP at rt; NF31/DMAP at 70 °C; NF31 at rt; NF31 at 70 °C.[18,20]

Figure 3. Resin appearance after treatment with NF31/DMAP:[20] top (from left to right): 2-chlorotritylchloride resin (Iris Biotech GmbH, 1.55 mmol g^{-1}), carboxy polystyrene (Novabiochem, 0.98 mmol g^{-1}), Merrifield resin (Novabiochem, 1.0 mmol g^{-1}); bottom (from left to right): aminomethylated polystyrene (Novabiochem, 1.1 mmol g^{-1}), HMPB–MBHA resin (Novabiochem, 0.51 mmol g^{-1}), NovaSyn® TG HMP resin (Novabiochem, 0.25 mmol g^{-1}), Kaiser oxime resin (Novabiochem, 1.3 mmol g^{-1}).



Scheme 1. Strategy for the evaluation of the NF31/DMAP test on primary and secondary alcohols, phenols and primary thiols (PG = protecting group).

Table 1
Results for the NF31/DMAP test on resins **3**–**6**[20]

Entry	Resin	x[a]	Loading[b] (μmol g^{-1})	XPG = OtBu (**3a** or **4a**)	XH = OH (**3b** or **4b**)	Entry	Resin	x[a]	Loading[b] (μmol g^{-1})	XPG = O*t*Bu (**5a**) or SMmt (**6a**)	XH = OH (**5b**) or SH (**6b**)
1	**3**	0.2	103.0 ± 5.4			7	**5**	0.2	124.5 ± 3.6		
2	**3**	0.05	14.89 ± 0.69			8	**5**	0.05	24.04 ± 1.88		
3	**3**	0.01	4.875 ± 0.400			9	**5**	0.01	3.692 ± 0.026		
4	**4**	0.2	123.1 ± 3.3			10	**6**	0.2	117.0 ± 13.0		
5	**4**	0.05	19.51 ± 1.10			11	**6**	0.05	24.14 ± 2.12		
6	**4**	0.01	2.829 ± 0.167			12	**6**	0.01	5.364 ± 0.503		

[a] x = number of equivalents of protected amino acid used for the coupling to aminomethyl polystyrene (see Scheme 1).
[b] Mean values (from at least 3 measurements) ± standard deviation.

phenol, or thiol functionality and (5) submission to the NF31/DMAP test (see Scheme 1).[23–26] The results of these experiments are listed in Table 1.

As evidenced by the enclosed pictures, the NF31/DMAP test is well suited for the sensitive detection of these amino acid side-chain functional groups with detection limits below 3–5 μmol g^{-1} in all cases. The primary alcohol and phenol function in serine and tyrosine (resins 3**b** and **5b**, entries 1–3 and 7–9) are very reactive toward NF31 in the presence of DMAP, as a bright red color is still observed even at the lowest (<1%) loading levels (the coloration can be entirely attributed to the alcohol functions as the resin-bound direct precursors **3a** and **5a** give colorless beads as shown). The secondary hydroxyl group in resin-bound threonine (resins **4b**, entries 4–6) is found to be less reactive; nevertheless, at a loading level of about 3 μmol g^{-1}, a pink color is still easily distinguished. Application of the NF31/DMAP test to cysteine resins **6b** (entries 10–12) again gives brightly red polymer beads, even at the lowest tested loading levels. However, of importance here is the lower stability of the dye-resin tethering due to the more labile thioester functionality formed during the labeling. It is therefore strongly recommended to omit any methanol washings, as the coloring agent in these cases is clearly released in solution, leading to an underestimation of the thiol concentration. A similar effect has been noted when visualizing the hydroxyl group on Kaiser oxime resin.

The presence of DMAP appears to be necessary: in all instances involving alcohol groups (resins **3b**–**4b**–**5b**), no coloration was observed using only NF31, even not at 70 °C (cf. Fig. 2). In the case of thiol-functionalized resins **6b**, the presence of DMAP is also useful, as only a faint pink color is observable in its absence. Our general protocol[20] is based on the optimized conditions for the detection of the sterically more hindered threonine secondary alcohol in **4b** at a challenging loading of <3 μmol g^{-1}. For this resin, while maintaining the low NF31 concentration, we found a concentration of 0.1 M DMAP to be necessary, leading to a clear coloration within 10 min, although a faint pink color is already visible even after 1 min.

In summary, a sensitive and highly practical colorimetric test for resin-bound alcohols, phenols, and thiols is reported. The protocol uses minimal amounts of commercially available reagents (as stable storable stock solutions) at room temperature and requires an overall time of only about 15 min (including sample preparation, staining reaction, bead washing and color inspection), offering the solid-phase chemist an interesting tool for monitoring these functional groups in synthetic transformations.

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23. *Preparation of resins* **3a**–**4a**–**5a**–**6a**: To an amount of aminomethylated polystyrene is added a solution of the required preactivated side-chain protected N^{α}-Fmoc-amino acid (subequivalent amounts, prepared by stirring equimolar amounts of amino acid, DIC and HOBt for 20 min in CH_2Cl_2/DMF 9:1), and the suspension is shaken for 24 h. The resin is filtered and washed consecutively with DMF, MeOH, and CH_2Cl_2. The remaining amino functions are capped by treatment with a 1:1:3 mixture of Ac_2O/DIPEA/CH_2Cl_2 (5 times 2 h) after which the resin is consecutively washed with DMF, MeOH, and CH_2Cl_2 and dried in vacuo. Complete capping of the amino functions is demonstrated by negative NF31 and NF31/DMAP tests. The amount of resin-bound amino acid is determined by UV-Fmoc-quantitation after treatment with a 20% 4-methylpiperidine solution in DMF (Ref. 24). The absorbance of the dibenzofulvene/4-methylpiperidine adduct at 300 nm is correlated to its concentration using a calibration line.
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26. *Preparation of resin* **6b**: An amount of resin **6a** (50 mg) is treated with 1 ml TFA/(iPr)$_3$SiH/CH_2Cl_2 1:5:94 for 3 h, after which the resin is filtered and washed consecutively with DMF, MeOH, and CH_2Cl_2 and subsequently dried in vacuo.