Colorimetric Tools for Solid-Phase Organic Synthesis

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Received February 2, 2004

One of the unresolved problems of solid-phase organic synthesis (SPOS) is the availability of general and rapid methods to monitor the transformation of functional groups present in molecules supported on insoluble supports. Color tests, far from providing the ultimate solution, may help in detection (and sometimes in quantification) of different functional groups. In this short review, we have collected most of the methods available and applied in SPOS with an Experimental Section that describes the procedure we have successfully applied to bead analyses in our laboratories.

Introduction

Combinatorial synthesis of small organic molecules can greatly enhance the capability to discover new chemical entities with a property of interest (such as new drugs).[1] Combinatorial strategies often employ a variety of resin-based technologies to improve synthetic efficiency, including the use of solid-phase organic synthesis (SPOS) as well as the application of polymer-supported reagents and quenching agents.[2] Notwithstanding the large popularity won by this technique, a critical step in the development of methods for SPOS is the assessment of the extent of completion of the reactions carried out on the polymeric matrix. For this reason, a great deal of effort has been dedicated over the years to find quick and reliable ways for monitoring solid-phase reactions.[3] NMR spectroscopy, such as high-resolution magic angle spinning,[4] gated decoupling NMR using ^{13}C-enriched molecules,[5] and the ^{19}F NMR technique[6] have proved to be powerful; however, these methods are not suitable for quick monitoring. Chromatographic techniques are not possible without cleavage from the support, and instrument techniques need the availability of expensive and sometimes dedicated equipment that are not always available. Thus, several analytical methods have been developed in order to obtain an effective reaction monitoring on-bead. Many color tests have been available in the past to detect functional group transformations, but many of them have been abandoned with the coming of spectroscopic techniques. Consequently, in the last several years, several researchers have developed (or rediscovered) simple colorimetric tests[7] that could allow the evaluation of the presence of functional groups on the resin-supported molecules.[8]

We have collected here most of the tests available in the literature, together with a detailed description of those we have successfully employed in our laboratories. We think that this collection will prove to be very useful to researchers involved in SPOS, in particular to those that are going to explore new reactions or new transformations on solid supports.[9] Obviously, those colorimetric tests are generally indicative and should be used exclusively to monitor the progress of the reaction, analytical techniques based on off-bead reaction monitoring remaining the most reliable method to quantify the outcome of an SPOS. Moreover, the identity of the final products must be always determined by cleavage and complete spectroscopic analysis. Finally, it must mentioned that in all the tests described, it is always advisable to carry out a blank control test.

Alkanes, Alkenes, Alkynes and Arenes. No specific tests are available because most of the reported libraries have been prepared on polystyrene−divinylbenzene resins. Although other supports, such as glass or cellulose, have been explored, we did not find any example of a test for detection of hydrocarbons.

Halogen Derivatives. The presence of halogens can be determined by microanalysis, if possible.[10] Although the method is sensitive, the amount of resin needed for the test is relatively high (at least 20 mg). When an activated halogen is present, nucleophilic displacement with a nuclephilic dye turns the bed the color of the dye. For instance, it is possible to use fluorescein or fuchsin (Scheme 1); in these cases, displacement of the halogen with the dye turns the beads yellow-green or yellow-green, respectively.[11]

Alternatively the reaction can be carried out using (*p*-nitrobenzyl)pyridine (PNBP) which turns the beads red under basic conditions[12] (Scheme 2).

These methods have been successfully applied to activated halogens, such as chloroacetyl esters and amides, benzyl chlorides, or allyl chlorides.[13] We have no experience in

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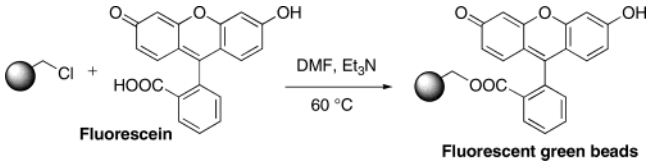
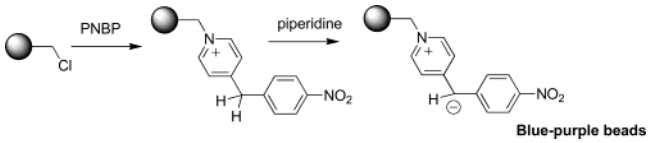
10.1021/cc049963a CCC: $27.50
Published on Web 06/25/2004

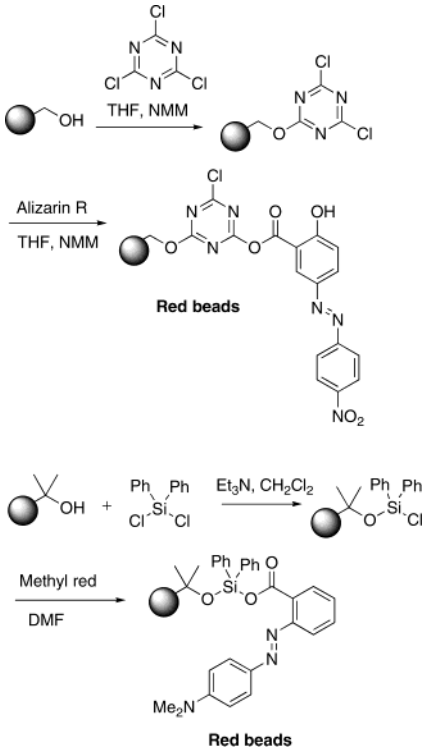
Scheme 1. Fluorescein Test



Scheme 2. PNBP Test



Scheme 3. TCT and Dichlorodiphenylsilane Tests for −OH

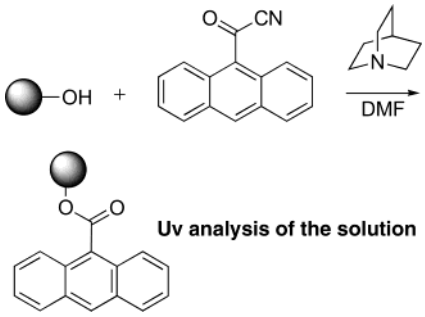


applying this method on hindered or secondary halogens which might give a competitive elimination.

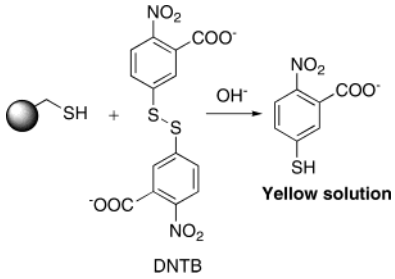
Alcohols. The presence of a hydroxyl group can be monitored by the formation of the tosylate, followed by displacement with PNBP and conversion of the resin-bound pyridinium salt to a strongly colored internal salt by treatment with base,[13] as previously described for detection of halogens. Amino and carboxyl groups give a negative test in these conditions. Full loading of Wang and Merrifield resins can be efficiently checked using this test.

In other methods, the −OH present on the resin is reacted with an excess of a reactive polychlorinated linker, such as 2,4,6-trichloro[1,2,5]triazine (TCT)[14] or diphenyldichlorosilane[15] (Scheme 3). After formation of the triazinyl or silyl ether, the residual reactive chloride undergoes nucleophilic substitution with a dye, such as alizarin R (red), fluorescein (fluorescent green), fuchsine (violet), or methyl red (red-orange), that paints the beads. The color can be observed under a simple microscope or even directly in the test tube. In both tests, carboxyl groups are expected to be positive. Because primary and secondary amines give a positive test

Scheme 4. Anthronylnitrile Test



Scheme 5. Ellman's Test



with TCT, this cannot be used to detect the presence of the −OH if these groups are present. Any group that is a stronger nucleophile than −OH might interfere in this test.

The absolute amount of an −OH group present on a resin can be determined with a procedure that can also be used as a fast qualitative "color" test.[16] The method is based on the rapid and efficient reaction of 9-anthroylnitrile with resin-bound hydroxyl groups (Scheme 4). A spectroscopic study of the concentration of the reagent determines the loading on the resin after subtracting the noncovalently absorbed amount of reagent molecules in the resin. Approximately 5−10 mg of resin and an analysis time of 30 min are needed. Most organic functional groups do not interfere with the analysis, except for the phenol group. Unhindered primary amines give the same reaction and can be also be quantitatively determined in this way. An alternative method for quantitative determination of the amount of OH present on the beads can be carried out by protection of the alcohol with dimethoxytrityl chloride followed by cleavage with acid and spectrophotometrical quantitative analysis of the released protecting group.[17]

Thiols. The presence of a free SH on the resin can be determined by Ellman's test based on the reaction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DNTB) with aliphatic thiols to generate a yellow anion that has a strong visible absorbance maximum at 412 nm ($\epsilon = 13600\ M^{-1}\ cm^{-1}$ in H_2O) (Scheme 5).[18] The UV absorbance of the solution can also be measured to determine the concentration of the thiol. The application of the Ellman's reagent for quantitative analysis of thiols supported on a wide range of solid supports has been reported.[19] A solution of the reagent in MeOH or MeOH/THF (1/1), basified with diisopropylethylamine (DIPEA), gave excellent results for a qualitative test, although satisfactory quantitative data were not obtained.

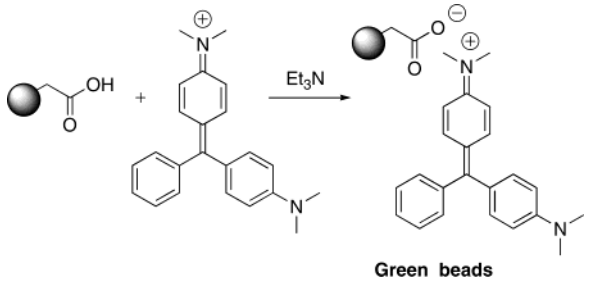
Further enhancements to the process can be achieved through a secondary treatment with excess dithiothreitol. This cleaves the disulfide bond and releases a second equivalent

Table 1. Correlation Chart between Tests and Functional Groups

	Fluorscein	PNBP	TCT	Ph_2SiCl_2	Anthronyl-	Ellman	Dansyhydrazone	*p*-Anhysald	Purpald	Fmoc-hydrazine
R−X (Cl, Br, I)	+	+	−	−	−	−	−	−	−	−
R−OH	−	+	+	+	+	−	−	−	−	−
R−SH	−	−	+	−/+	+	+	−	−	−	−
R−COOH	−	−	+	−	−	−	−	−	−	−
RCHO	−	−	−	−	−	−	+	+	+	+
R−CO−R	−	−	−	−	−	−	+	+	+	+
RNH_2	−	−	+	−	+	−	−	−	−	−
R_2NH	−	−	+	−	±	−	−	−	−	−
R_3N	−	−	−	−	−	−	−	−	−	−
$RNH-NH_2$	−	−	+/−	−	−	−	−	−	−	−
RN_3	−	−	−	−	−	−	−	−	−	−

	Kaiser	Chloroanil	β-Naphthol	$NF\text{-}31_2$	DABITC	NPIT	Blue Bromophenol	Picric Acid	TNBS
R−X (Cl, Br, I)	−	−	−	−	−	−	−	−	−
R−OH	−	−	−	−	−	−	−	−	−
R−SH	−	−	−	−	−	−	−	−	−
R−COOH	−	−	−	−	−	−	−	−	−
RCHO	−	−	−	−	−	−	−	−	−
R−CO−R	−	−	−	−	−	−	−	−	−
RNH_2	+	−	+	+	+	+	+	+	+
R_2NH	−	+	+	+	+	+	+	+	+
R_3N	−	−	−	−	−	−	+	+	+
$RNH-NH_2$	+/−	+/−	−	+	+	+	+	+	+
RN_3	+(PPh_3)	−	−	−	−	−	−	−	−

Scheme 6. Malachite Green Test



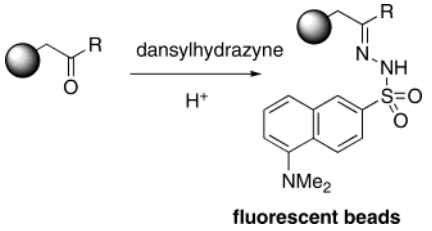
of thionitrobenzoate, allowing a duplicate value to be obtained from the same resin sample.[20]

Carboxylic Acids. The presence of a carboxylic acid (or another ionizable group) on the resin can be detected by deprotonation with Et_3N in DMF followed by formation of a salt with a positively charged dye. Malachite Green (Table 1) is an effective indicator of the presence of an acid on polystyrene resins[21] (Scheme 6).

The test is very sensitive (1% of COOH on the resin can be detected), and in the presence of free COOH, the beads are colored dark green; a negative answer is indicated by colorless gel beads. The color of the beads can be observed with a microscope (10× magnification is enough) or even with the naked eye. In addition, aromatic and substituted carboxylic acids or hydroxamic derivatives can be easily detected. The method based on 9-anthroylnitrile, previously described for alcohols, can also be used for carboxylic acids and phenols.[17]

Aldehydes and Ketones. Aldehydes and ketones can be assayed by conversion into the corresponding fluorescent dansyl hydrazone. This method is simple to perform and can be used to determine the absolute amount of aldehydes and aliphatic ketones on resin (Scheme 7). It can be applied using 3−10 mg of resin sample (for loadings 1.0−0.3 mmol/g) containing low amounts of functional groups.[22]

Scheme 7. Fluorescent Test for Carbonyl Compounds

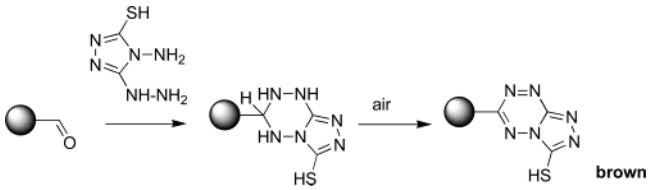


Iron (III) is able to produce an intensely colored complex (rust-brown) with enolizable ketones. Therefore, this old colorimetric assay is suitable and sensitive enough to detect the presence (or absence) of β-dicarbonyl compounds on solid support.[23]

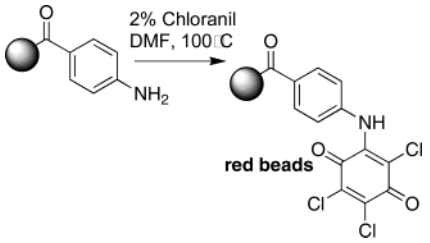
Aromatic and aliphatic aldehydes can be detected by reaction of the beads with an ethanol solution of *p*-anisaldehyde, sulfuric acid, and acetic acid.[24] After heating, the beads turn dark red-violet. To determine the sensitivity of the method, several BAL (backbone amide linker) resins were prepared by limited incorporation of the handle to the resin. Depending on the amount of aldehyde present on the resin, the resin with the *p*-anisaldehyde solution becomes orange to red after treatment. To check the compatibility of this test with more acid-labile resins, such as Wang and chlorotrityl resins, the BAL handle was incorporated into both resins through an ester bond, and samples were submitted to the *p*-anisaldehyde test. Whereas chlorotrityl resins underwent cleavage under these conditions, Wang-type linkers were stable. The *p*-anisaldehyde test is compatible with the presence of other functional groups, such as amines or hydroxyl groups.

A sensitive and specific color test for the detection of the presence of resin-bound aldehyde groups using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) has been recently reported[25] (Scheme 8). Aldehyde-containing resins turn

Scheme 8. Purpald Test



Scheme 9. Chloranil Test



dark-brown to purple after a 5-min reaction with Purpald followed by a 10-min air oxidation period. Resins that possess other functional groups (i.e., ketone, ester, amide, alcohol, and carboxylic acid) do not change color under the same conditions. The detection limit is 20 μmol/g for polystyrene-based aldehyde resins.

The intensity of the color depends on the loading of the resin as well as the aldehyde structure. Swelling of the resin was necessary, and DMF was found to be the best solvent. The test is specific for aldehydes, because resin-bound ketones did not react. Ester, acid, amide, alcohol, and amine groups all gave negative results under the same conditions. Water-compatible resins, such as NovaGel and TentaGel, showed even better reactivity.

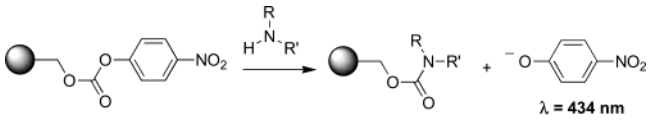
Aldehydes and ketones may also be quantitatively assayed by conversion to the Fmoc hydrazone, removal of the Fmoc group by piperidine/DMF, and UV spectroscopic determination of the dibenzofulvene adduct.[26] This method is easy to perform and accurately quantifies the absolute amount of aldehyde and aliphatic ketone groups on resin. It can be applied using 3−10 mg of resin sample (for loadings 1.0−0.3 mmol/g) containing low molar amounts of functional groups. It is not suitable for acid-labile linkers.

Amines. Because the amino group is ubiquitous in solid-phase peptide synthesis, several methods are available to monitor the presence of amines on bead.

1. Primary Aliphatic Amines. The most popular colorimetric assay for primary aliphatic amines is the ninhydrin test (Kaiser test).[27] In this test, the beads are treated with two solutions containing ninhydrin and phenol, respectively. After heating, the presence of the amine is indicated by a blue solution and blue beads. For a complete reaction, the two conditions must be fulfilled. The ninhydrin reaction can also be employed for quantitative monitoring.[28]

2. Primary Aromatic Amines. Aromatic amines on a solid support can be detected by treatment with a 2% solution of chloranil in DMF that produces a change in the solution from bright yellow to an intensive brown or violet color, whereas the beads turn red, within 5 min at 100°C[29] (Scheme 9). The chloranil test can be applied to a very small sample of the resin (~1 mg).

Scheme 10



To detect free primary aromatic amines on cellulose beads, a "β-naphthol test" can be also used.[30] The solid support treated at 0°C first with an acid aqueous solution of $NaNO_2$ and then with a basic aqueous solution of β-naphthol immediately changes color from white to red. This rather sensitive assay enables the detection of even small amounts of supported primary aromatic amines.

3. Secondary Aliphatic Amines. The determination of a secondary aliphatic amine can be carried out with the chloranil test.[31] Indeed, this test is positive with primary amines, too, but used after that, a negative Kaiser test ensures the absence of free $-NH_2$ groups. Alternatively, primary or secondary amines can be reacted with a colored reagent that forms a functional derivative and paints the beads with the reagent color. This is the case of NF-31,[32] DABITC (4-dimethylaminoazobenzene-4′-isothiocyanate),[33] or NPIT (nitro phenylisothiocyanate),[34] that was developed to monitor less reactive and sterically hindered primary or secondary amines.

Quantification of amines (primary or secondary) can be carried out on the N-Fmoc-protected compounds. Upon Fmoc cleavage, the resulting piperidine/dibenzylfulvene adduct can be quantitatively determined by its absorbance at 301 nm.[35]

4. Tertiary Aliphatic Amines. A basic amine can be assessed by acid/base titration using a suitable indicator or reagent, such as bromophenol blue,[36] picric acid,[37] or TNBS (2,4,6-trinitrobenzensulfonic acid).[38]

Other Nitrogen-Containing Functional Groups. Hydrazine derivatives supported on resin can be determined using *p*-dimethylaminobenzaldehyde in alkaline media.[39]

Aliphatic azides could be detected by azide reduction to amine by a phosphine, followed by (or concurrent with) treatment with the Kaiser test reagents.[40] Indeed, the simple addition of triphenylphosphine and water to the standard Kaiser test protocol provided a selective indicator for the presence of azides in solution, on TLC plates, or when supported on resin beads.[41] The tolerance of the azide detection method toward other functional groups is high, being compromised only by fast oxidation of the phosphine or quenching of the generated amine.

Finally, a rapid and quantitative test for monitoring the yield of coupling of amino compounds on polystyrene resins through a carbamate linker has been developed.[42] In addition to their efficiency in promoting the anchoring of amino moieties, the activated *p*-nitrophenyl carbonate resins, releasing *p*-nitrophenate in a basic solution, afford a valuable chromophore for quantitatively monitoring the efficiency of the coupling (Scheme 10).

The method can be used both for loading estimation of derivatized resins and for kinetic studies.

Resin-Bound Indicators. A new concept for solid-phase synthesis is that of "self-indicating" resins containing resin-bound indicators that act as integral sensors for the chemistry being carried out. Resin-bound indicators allow the detection of amines both in solution and on the solid phase. The resin-

bound dye can be used as a self-indicator for in situ monitoring of solid-phase peptide synthesis as well as array library synthesis.[43] Since the newly developed self-indicating resins are able to follow coupling reactions at the level of individual beads (instead of checking reaction completion globally), this resin should be helpful during mix-and-split synthesis in which every bead may contain a different peptide sequence and, therefore, exhibit different reaction kinetics. It also offers the possibility of monitoring the release of free amines on the resin during chemical or biological screening.

The dye bromophenol blue (3′,3″,5′,5″-tetrabromophenolsulfophthalein) was chosen as an indicator because of its evident color change from yellow to dark blue and its high extinction coefficient, thus giving high sensitivity.[44] The resin was successfully used as a "sensor" for monitoring solid-phase peptide synthesis and was applied for in situ reaction monitoring during the synthesis of a library of ureas in a highly successful manner.

The dansyl group has been also used to protect supported alcohols for the fluorescent labeling of the resin.[45]

In conclusion, the presence of many important functional groups can be assessed using a rapid colorimetric spot test. The use of these tests and the use of on-bead FT-IR may solve many issues for a correct qualitative analysis of the reaction progress on solid phase.

Experimental Section

This section contains the procedures for colorimetric detection of several functional groups that we have carried out in our laboratories which have given reliable results. For other procedures see the original literature.

Colorimetric Test for Halogens. (1) Sample a few beads of the resin, and wash them several times with the reaction solvent and DMF. Transfer the beads to a test tube, and add 1 mL of DMF. (2) Add 1 drop of Et_3N and 1 mg of fluorescein. (3) Mix well and heat at 60 °C for 15 min. (4) Remove the solution, and wash the beads with DMF until the solvent is clear. Wash with THF and Et_2O. (5) Observe the color under the microscope. A positive test is indicated by brilliant yellow-green beads.

Colorimetric Test for Alcohols (and Phenols). (1) Sample a few beads of the resin, and wash them several times with the solvent of the reaction and DMF. Transfer the beads to a test tube, and add 5 mL of THF. (2) Add 1−2 drops of *N*-methyl morpholine (NMM) and 1−2 mg of TCT. (3) Shake at room temperature for 10 min. (4) Remove the solution, and wash the beads with THF and DMF. (5) Add 2 mL of THF, 1−2 drops of NMM, and 1 mg of Alizarin R (or another nucleophilic dye, such as fuchsin), and shake for 10 min. (6) Remove the solution, and wash the beads with DMF until the solvent is clear. Wash with THF and Et_2O. (7) Observe the color under the microscope. A positive test is indicated by red (or fuchsia) beads.

Colorimetric Test for Thiols. (1) Dissolve DNTB in a pH 8 buffer solution (∼10 mL of a 0.1 M solution). (2) Sample a few beads of the resin, and wash them several times with the solvent of the reaction and DMF. Transfer the beads to a test tube, and add 2 mL of MeOH. (3) Add 0.5 mL of the solution of DNTB, and leave the mixture for 15 min. (4) A positive test is indicated by a deep yellow color of the solution.

Colorimetric Test for Carboxylic Acids. (1) Sample a few beads of the resin, and wash them several times with the solvent of the reaction and DMF. Transfer the beads to a test tube, and add 1 mL of MeOH. (2) Add 1 mL of a 0.025 solution (w/w) of Malachite green, followed by 1 drop of Et_3N. (3) Mix well for 5 min. (4) Remove the solution, and wash the beads with EtOH until the solvent is clear. Wash with THF and Et_2O. (5) Observe the color under the microscope. Dark green beads indicate a positive test.

Colorimetric Tests for Aldehydes. (1) Prepare a solution with H_2SO_4 (9 mL), EtOH (88 mL,) CH_3COOH (1 mL), and *p*-anisaldehyde (2.55 mL). This solution must be used immediately after preparation. If protected from light and stored at 0°C, it may be used for a few additional days. (2) Sample a few beads of the resin, and wash them several times with the reaction solvent and MeOH. (3) Add 2−3 drops of the solution of *p*-anisaldehyede, warming the test tube at 110 °C. After 4 min, discharge the solution, and wash the beads with MeOH until the solution is clear. (4) Observe the color of the beads. The test is positive when the beads are dark purple.

Colorimetric Tests for Amines. 1. Primary Amines: Kaiser Test. (1) Prepare two solutions containing for solution A, 1 g of ninhydrin in 20 mL of ethanol; and for solution B, 40 g of phenol (use phenol of purity higher than 95%) in 10 mL of ethanol. (2) Sample a few beads of the resin, and wash them several times with the reaction solvent, DMF, and MeOH. The MeOH is removed almost completely with a pipet. (3) Add 2 drops of solution A, 2 drops of solution B, and 2 drops of pyridine. (4) Heat the test tube with an oil bath at 110 ± 5 °C for 2 min. (5) Observe the color of the solution and of the beads (under the microscope). A blue solution and red-violet beads indicate a positive test. The test is negative if the solution is blue and the beads are yellow or the solution is yellow and the beads are light blue.

2. Secondary Amines (Chloranil Test). (1) Prepare two solutions containing for solution A, 2% (v/v) acetaldehyde in DMF; and for solution B, 2% (w/w) chloranil (2,3,5,6-tetrachloro-1,4-benzoquinone) in DMF. (2) Sample a few beads of the resin, and wash them several times with the solvent of the reaction, DMF, and MeOH. The MeOH is removed almost completely with a pipet. (3) Add 2 drops of solution A and 2 drops of solution B. (4) Allow to stand at room temperature for 5 min. (5) Observe the color of the solution and of the beads (under the microscope). A positive test is indicated by iron-grey-blue beads.

3. Tertiary Amines (Bromophenol Blue). (1) Samplea few beads of the resin, and wash them several times with the solvent of the reaction, DMF (free from amines), and CH_2Cl_2. (2) Add 2 drops of a 1% solution of bromophenol blue in dimethylacetamide. (3) The test is positive if the color of the beads changes immediately from yellow-green into dark blue.

Colorimetric Test for Azides. (1) Sample a few beads of the resin (at least 1 mg), and wash them several times with the reaction solvent, followed by DMF. (2) Add 2 drops of a 5% (w/w) solution of PPh_3 in THF, followed by 2 drops

of H_2O, 2 drops of a 5% solution of ninhydrin in EtOH, 1 drop of pyridine, and 1 drop 80% phenol in EtOH. (3) Warm the tube in a sand bath at 200 °C for 5 min. Blue solution and red-violet beads indicate a positive test.

Acknowledgment. We thank MIUR (Rome), NiKem Research (Milan), and Siena Biotech (Siena) for financial support.

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CC049963A