A Novel Sensitive Colorimetric Assay for Visual Detection of Solid-Phase Bound Amines

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A new simple and efficient method for the detection of incomplete coupling reactions during solid-phase peptide synthesis is decribed. Using *p*-nitrophenyl ester **1** (NF31), free amino groups can be visually detected on the resin by direct coloring of the beads. A specific feature of the assay resides in the possibility of detection of sterically hindered primary amines.

Introduction

The development of chemical libraries through combinatorial techniques is largely responsible for a spectacular revival in solid support synthesis.[1][2] Indeed, solid-phase synthesis is the preferred tool in the construction of large libraries, in particular when the popular split-mix methodology is used.[3] Since the amplitude of a library depends in the first place on the number of coupling steps in the process, it is essential that every coupling step should occur in (almost) quantitative yield. Hence the possibility of evaluating the completeness of a coupling step is of critical importance.

In the chemical sense, a coupling step should involve a reliable, fast, and general reaction, preferably adaptable to automation. Among the various possibilities, the formation of peptide bonds involving amino acids has been the preferred coupling process. It may therefore be somewhat surprising that there still exists a need for new improved methods for monitoring coupling efficiency in a simple and reliable way. This is primarily because the quest for more diversity in the construction of libraries has led to the selection of non-natural amino acids, including secondary and hindered primary amines, for incorporation in the basic coupling set. The present work describes a novel sensitive method for detection of free amines on the solid phase which also allows for monitoring peptide couplings.

The problem of evaluating the effectiveness of a coupling step can in principle be addressed in several ways: (1) by detachment of the molecule from the solid support followed by full characterization of the product(s); (2) by direct spectroscopic identification of the resin-bound molecule;[4] or finally, (3) by the use of one of the several available qualitative or quantitative assays.[5] In the latter context a brief overview of the more common methods is presented in Table 1.[6–15] The methods can be subdivided into three categories: (1) The presence of unchanged amine is assessed by acid/base titration; in particular using a suitable indicator, such as picric acid[9] or bromophenol blue.[11] (2) Conversion is evaluated in a two-step process involving attachment of a reporter group to any left amine followed by selective detachment and quantitation; the spectroscopic measurement of the concentration of dimethoxytrityl (DMT) cation lies at the basis of the DMT[12] and the "nitrophenylisothiocyanate-*O*-trityl" (NPIT) test.[14] This method bears some resemblance with the cleavage and quantitation of Fmoc protective groups after coupling.[16] (3) The presence of free amine is revealed by an (invasive) colorimetric assay with visual detection as the most simple option; e.g. the standard Kaiser ninhydrin test,[7] the increasingly used 2,4,6-trinitrobenzenesulphonic acid (TNBS) tests,[10] and the recently described color reagent "4-*N,N′*-dimethylaminoazobenzene-4′-isothiocyanate" (DABITC).[15]

The latter tests however are not devoid of shortcomings. In particular, the ninhydrin test cannot be used for derivatisation of proline;[17] the TNBS test gives erroneous results in the case of sterically hindered amines (vide infra) and the DABITC reagent, yielding a yellow or orange colored bead, is not reliable since resins often become yellow or orange when exposed to chemicals. In this paper we wish to report on the development and use of a simple, rapid and sensitive visual method for the detection of amines bound to solid support using activated ester **1** (NF31).

Results and Discussion

The development of **1** is the result of a search for a reactive, yet stable, acylating agent containing a chromogenic acyl part. The choice for Disperse Red 1 was based on the following: the azo unit is reasonably chemically inert (at least under non-oxidizing conditions), and among the several substituted types only a few possess a λ_{max} larger than 450 nm such as Disperse Red 1 (λ_{max} 503 nm).[18] The *p*-

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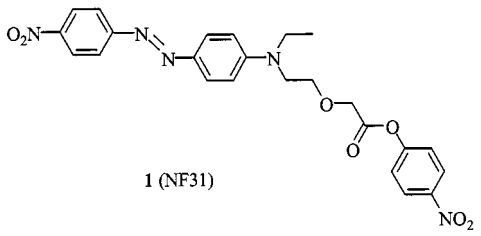
Table 1. Overview of monitoring methods for SPPS[a]

Test	Features
Pyridine · HCl[b]	quantitative detection of free amines in Boc-SPPS by $AgNO_3$ titration
Ninhydrin	visual detection (blue) of free amines
→ Kaiser[c]	quantitative determination by UV
→ Sarin[d]	
Picric acid[e]	quantitative determination of free amines by acid-base titration followed by UV measurement
TNBS[f]	visual detection (orange) of primary amines
Bromophenol blue[g]	visual detection (blue) of free amines by acid-base titration
DMT[h]	quantitative determination of amines and alcohols by a tritylation-detritylation procedure followed by UV measurement
Chloranil[i]	visual detection (blue) of secondary amines
NPIT[j]	qualitative and quantitative determination of amines by a derivatisation−detritylation procedure followed by UV-measurement
DABITC[k]	visual detection (yellow to orange) of primary and secondary amines

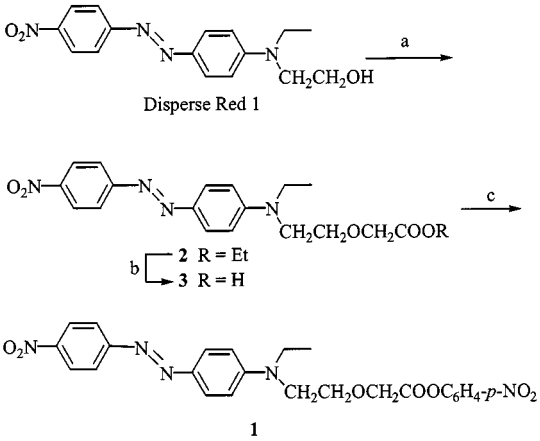
[a] The different entries are given in chronological order. − [b] See ref.[6] −[c] See ref.[7] − [d] See ref.[8] − [e] See ref.[9] − [f] See ref.[10] − [g] See ref.[11] −[h] See ref.[12] − [i] See ref.[13] − [j] See ref.[14] − [k] See ref.[15]



1 (NF31)

nitrophenyl ester **1** is readily accessible from commercial Disperse Red 1 by a high-yielding three-step procedure (Scheme 1, 51% overall yield) involving (a) treatment with ethyl diazoacetate,[19] (b) saponification, and (c) $POCl_3$ condensation with *p*-nitrophenol.[20] The product is purified by precipitation from a mixture of acetone, diethyl ether, and pentane. Reagent **1** can be stored as such at room temperature for several months. It is soluble in a variety of solvents such as dichloromethane (DCM), acetonitrile, acetone, methanol, and pyridine. Solutions of **1** in DCM or acetonitrile can be stored for several months at −18°C.

Exposure of resin beads containing free amino groups to **1**, followed by a thorough washing procedure, leads to intensely red colored beads. In contrast beads exposed to pure Disperse Red 1 remain colorless, indicating that the coloring does not result from the mere adsorption of the reagent by the resin. The nature of the covalent bonding was assessed in the following way: TentaGel−NH_2 was shaken overnight at room temperature in DCM in the presence of **1**. After thorough washings (DMF, MeOH, DCM) until a colorless filtrate was obtained, the red acylated beads were dried and analyzed by ^{13}C NMR; the signals corresponding to the acyl part of **1** are clearly distinghuished; especially the resonances at low field are characteristic: δ = 173.4, 128.0, 126.9, 124.8, 113.8.



Scheme 1. Synthesis of the *p*-nitrophenyl ester **1** (NF31). a) $N_2CHCOOEt$, CH_2Cl_2/toluene, 1 h at 40°C, 12 h at room temp., 57%; b) KOH, MeOH, reflux, 1.5 h, 97%; c) $POCl_3$, pyridine/CH_2Cl_2, *p*-nitrophenol, rt, 4 h, 93%

The following procedure has been developed for monitoring the completeness of a peptide coupling reaction on solid phase. A few beads are suspended in 100 μL of a 0.002 M solution of **1** in acetonitrile. After heating at 70°C during 10 minutes, the beads are washed with DMF (3×), MeOH (3×) and DCM (3×). Beads containing free amino functions appear as red spheres while completely coupled beads (containing no free amino groups) remain colorless.[21] The experimental conditions of the above procedure are not critical: e.g. lower temperature and longer reaction times will yield the same result (overnight at room temperature). The test can also be performed in DCM. So far, solid phases that have been used include TentaGel−NH_2 resin and Polystyrene Wang resin.

Obviously the sensitivity of the assay is one of the most critical features. This was evaluated in two ways. (1) A series of mixtures containing Wang−Gly−NH_2 and Wang−Gly−Fmoc in varying proportions was prepared, the amount of free amine being varied from 20% up to 0.5%. Each sample was treated with **1** (0.01 M in DCM; 10 minutes at 70°C) followed by thorough washing. Visual perception of colored beads is straightforward even in the last case (Figure 1). Obviously, in this test the concentration of free amine on a single bead is high.

A more realistic test is related to the detection of free amine evenly distributed on the solid support. (2) Therefore a series of resin samples of known free amine content was prepared by treatment of Wang−Gly−NH_2 with a mixture of Fmoc−Ala and Boc−Ala in varying proportions. After selective removal of the Fmoc-protective group beads were obtained with a free amine content of 50, 20, 10, 5, and 2%. The different fractions were treated with both **1** and TNBS. Figure 2 allows for visual comparison of both assays at different sensitivity levels (50% to 2%).

A major characteristic of **1** is its high reactivity even with sterically hindered amines. The following experiment illustrates the point. Derivative **4** (Figure 3) was obtained by coupling of TentaGel−NH_2 resin with a novel steroid-based scaffold for combinatorial chemistry,[22] which we are

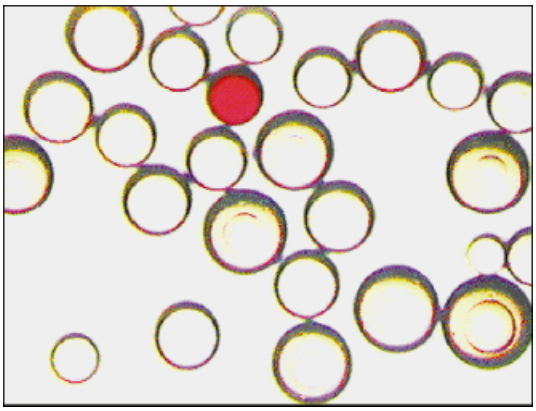


Figure 1. Photograph of part of a resin mixture containing 0.5% of beads with free amino groups after treatment with NF31

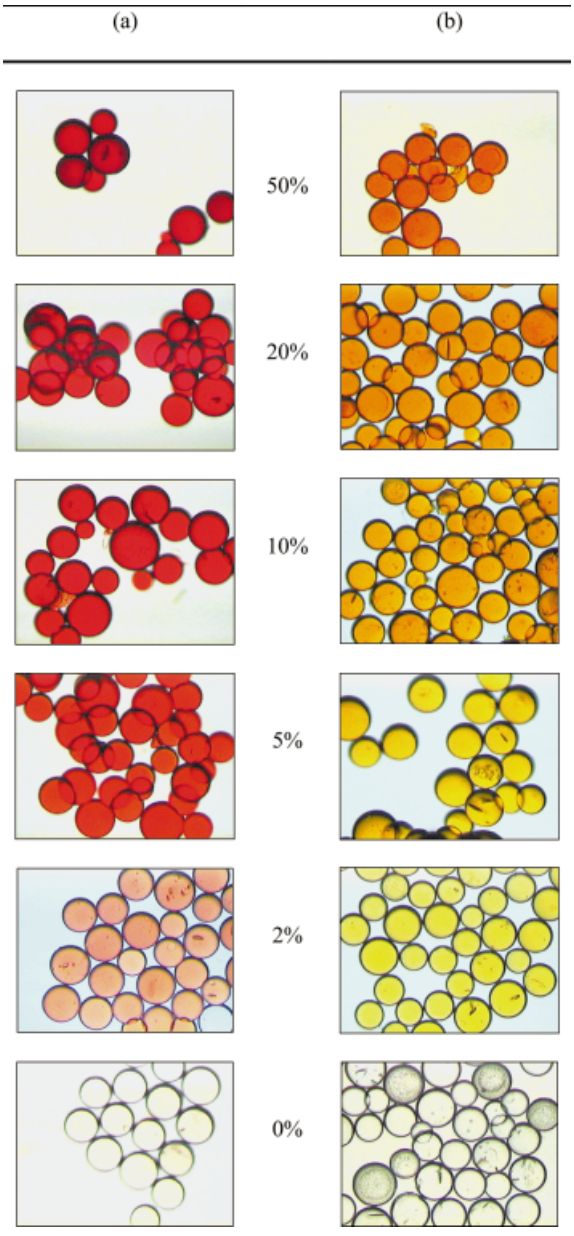
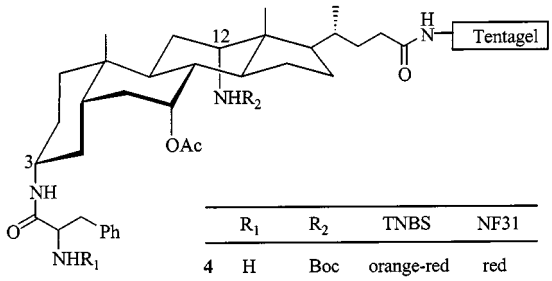


Figure 2. Photographs of resin beads with different amine content (given in%) after treatment with: (a) NF31; (b) TNBS

presently using in the context of the development of a library of hydrolase mimics.



	R_1	R_2	TNBS	NF31
4	H	Boc	orange-red	red
5	Ac	Boc	colorless	colorless
6	Ac	H	colorless	red

Figure 3. Result of visual comparison of NF31 and TNBS assays on a resin-bound sterically hindered primary amine

The free amino function on position 3 in **4** reacts with the TNBS reagent yielding orange beads. However, after capping of the free amine and deprotection of the Boc-protected amine **5** on position 12, subsequent treatment with TNBS reagent in the usual way did not lead to the development of the characteristic orange color on the beads. Presumably the steric hindrance in **6** is too large for the nucleophilic aromatic substitution to take place and the beads remain colorless. In contrast treatment of the same resin **6** with reagent **1**, followed by washing as described above, resulted in intensely red colored beads, in accord with the presence of the free amino group. Another feature of the currently described method lies in the possibility of following coupling reactions to proline. Whereas the ninhydrin[23] and TNBS[24] tests give negative results with proline, treatment of TentaGel−Pro−NH with reagent **1** leads to the characteristic red beads.

Conclusion

The visual detection with reagent **1** (NF31) is an efficient and sensitive method to monitor coupling reactions involving free amines on solid phase, and is especially valuable for detection of sterically hindered amines

Experimental Section

General: Thin-layer chromatography was performed on Merck silica gel 60F-254 TLC plates. − IR (NaBr): Perkin−Elmer 1600 series. − ^{1}H NMR ($CDCl_3$): 500 MHz − Bruker AN-500 (internal TMS as reference). − ^{13}C NMR ($CDCl_3$): 50 MHz − Varian Gemini-200 (with DEPT program). − MS: Finnigan 4000 or Hewlett−Packard 5988A. − Photographs were taken using a Leitz microscopic camera, laborlux 12 Pol S, equipped with a JVC color video camera. − TentaGel−NH_2 resin (90 µm, 0.19 mmol/g) and Polystyrene PHB (Wang resin, 100−200 mesh, 1.1 mmol/g) were purchased from Rapp Polymere GmbH (Tubingen, Germany). − Amino acids were obtained from Novabiochem. − Solvents were distilled with drying agents under nitrogen atmosphere: Et_2O and toluene with sodium; dichloromethane (DCM) and pyridine with

CaH_2. *N,N*-dimethylformamide (DMF) extra dry was purchased from Biosolve and used without further purification. Disperse Red 1 was purchased from Aldrich Chemical Company.

Ethyl 5-{*N*-Ethyl-*N*-[4-(4-nitrophenyl)azo]phenyl}amino-3-oxapentanoate (2): To a solution of Disperse Red 1 (6.28 g, 20 mmol) and $Rh_2(OAc)_4$ (150 mg, 0.34 mmol) in a mixture of DCM (100 mL) and toluene (100 mL) was added at 40°C a solution of ethyl diazoacetate (8.4 mL, 80 mmol) in toluene (40 mL) over a one-hour period. The reaction mixture was then stirred overnight at room temperature. Half of the solvent was removed under reduced pressure and the residue was purified by chromatography over a 10 cm diameter G2 fritted disc filtering column filled with silica gel, using a gradient of 10% to 25% EtOAc in toluene.[25] The fractions containing the desired compound were concentrated to 20 mL and to the warm solution was slowly added 200 mL of pentane. After refrigeration overnight the precipitate was filtered and washed with 10% Et_2O in pentane and dried, yielding 4.56 g (57%) of a bright red powder, m.p. 80°C. − R_f = 0.75 (EtOAc/toluene, 1:1). − IR (NaBr): $\tilde{\nu}$ = 2974 cm^{-1}, 2902, 1759, 1603, 1518, 1392, 1340, 1209, 1138, 1106, 1032, 964, 858, 826. − ^1H NMR (500 MHz, $CDCl_3$): δ = 8.32 (2 H, m), 7.92 (2 H, m), 7.89 (2 H, m), 6.78 (2 H, m), 4.22 (2 H, q, *J* = 7.1 Hz), 4.11 (2 H, s), 3.78 (2 H, t, *J* = 6.0 Hz), 3.69 (2 H, t, *J* = 6.0 Hz), 3.57 (2 H, q, *J* = 7.1 Hz), 1.28 (3 H, t, *J* = 7.1 Hz), 1.25 (3 H, t, *J* = 7.1 Hz). − ^{13}C NMR (50 MHz, $CDCl_3$): δ = 170.1, 156.8, 151.4, 147.3, 143.6, 126.3, 124.7, 122.6, 111.3, 69.0, 68.7, 60.9, 50.1, 46.0, 14.2, 12.2. − ESMS; *m/z* (%): 401 (30) $[M + H]^+$, 423 (100) $[M + Na]^+$.

5-{*N*-Ethyl-*N*-[4-(4-nitrophenyl)azo]phenyl}amino-3-oxapentanoic Acid (3): To a solution of **2** (5 g, 12.5 mmol) in MeOH (300 mL) and toluene (70 mL) was added KOH (4.062 g, 62.5 mmol) and the resulting reaction mixture was refluxed under nitrogen for 1.5 hours. The mixture was concentrated to 50 mL under reduced pressure, acidified with 25 mL of 10% HCl and further diluted with 120 mL of H_2O. The aqueous phase was extracted four times with DCM, the combined organic extracts were washed with H_2O and dried with $MgSO_4$. After concentration to 25 mL, 120 mL of diethyl ether were slowly added and the mixture was refrigerated overnight after which the desired product precipitates as a dark-red powder (4.5 g, 12.097 mmol, 96.7%), m.p. 161°C. − R_f = 0.08 (EtOAc/toluene, 1:1). − IR (DCM): $\tilde{\nu}$ = 2925 cm^{-1}, 2853, 1721, 1596, 1513, 1385, 1337, 1239, 1135, 1104, 855, 824, 668. − ^1H NMR (500 MHz, $[D_6]$DMSO): δ = 8.36 (2 H, m), 7.93 (2 H, m), 7.83 (2 H, m), 6.89 (2 H, m), 4.08 (2 H, s), 3.68 (2 H, m), 3.65 (2 H, m), 3.55 (2 H, q, *J* = 7.0 Hz), 1.16 (3 H, t, *J* = 7.0 Hz). − ^{13}C NMR (50 MHz, $[D_6]$DMSO): δ = 171.8 (C=O), 156.3 (Ar), 151.8 (Ar), 146.8 (Ar), 142.7 (Ar), 126.2 (Ar), 125.1 (Ar), 122.6 (Ar), 111.6 (Ar), 68.2 (CH_2), 67.9 (CH_2), 49.5 (CH_2), 46.3 (CH_2), 12.0 (CH_3). −ESMS; *m/z* (%): 373 (100) $[M + H]^+$.

4-Nitrophenyl-5-{*N*-ethyl-*N*-[4-(4-nitrophenyl)azo]phenyl}amino-3-oxapentanoate (1): To a solution of **3** (2.322 g, 6 mmol) and *p*-nitrophenol (0.834 g, 6 mmol) in pyridine (100 mL) and DCM (120 mL) at −15°C was added a solution of $POCl_3$ (1.006 mL, 10.8 mmol) in DCM (10 mL) over a one-hour period. The temperature was slowly raised to room temperature. After 4 h,s the reaction mixture was poured into a mixture of DCM (250 mL) and ice cold H_2O (200 mL). The aqueous phase was extracted two times with DCM and the combined organic layers were washed with a sodium bicarbonate solution and with H_2O. After drying with $MgSO_4$, the solvent was removed in vacuo and the residue dissolved in warm acetone (50 mL). After diluting with diethyl ether (20 mL), pentane (150 mL) was added very slowly. Refrigeration overnight, filtration, and washing with pentane, yielded 2.745 g (5.56 mmol, 92.8%) of **1** as an intense red powder, m.p. 125°C. − R_f = 0.70 (EtOAc/toluene, 1:1). − IR (KBr): $\tilde{\nu}$ = 2964 cm^{-1}, 1778, 1604, 1588, 1522, 1389, 1337, 1260, 1206, 1159, 1120, 854, 818, 804. − ^1H NMR (500 MHz, $CDCl_3$): δ = 8.33 (2 H, d, *J* = 8.9 Hz), 8.28 (2 H, d, *J* = 9.1 Hz), 7.92 (2 H, d, *J* = 8.9 Hz), 7.89 (2 H, d, *J* = 9.2 Hz), 7.29 (2 H, d, *J* = 9.1 Hz), 6.79 (2 H, d, *J* = 9.2 Hz), 4.41 (2 H, s), 3.88 (2 H, t, *J* = 5.8 Hz), 3.74 (2 H, t, *J* = 5.8 Hz), 3.59 (2 H, q, *J* = 7.1 Hz), 1.27 (3 H, t, *J* = 7.1 Hz). − ^{13}C NMR (50 MHz, $CDCl_3$): δ = 167.9, 156.7, 154.6, 151.2, 147.3, 145.5, 143.6, 126.2, 125.3, 124.7, 122.6, 122.2, 111.3, 69.4, 68.4, 50.1, 46.1, 12.2. − ESMS; *m/z* (%): 494 (100) $[M + H]^+$, 516 (40) $[M + Na]^+$.

Acknowledgments

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