



## NPIT: A NEW REAGENT FOR QUANTITATIVELY MONITORING REACTIONS OF AMINES IN COMBINATORIAL SYNTHESIS

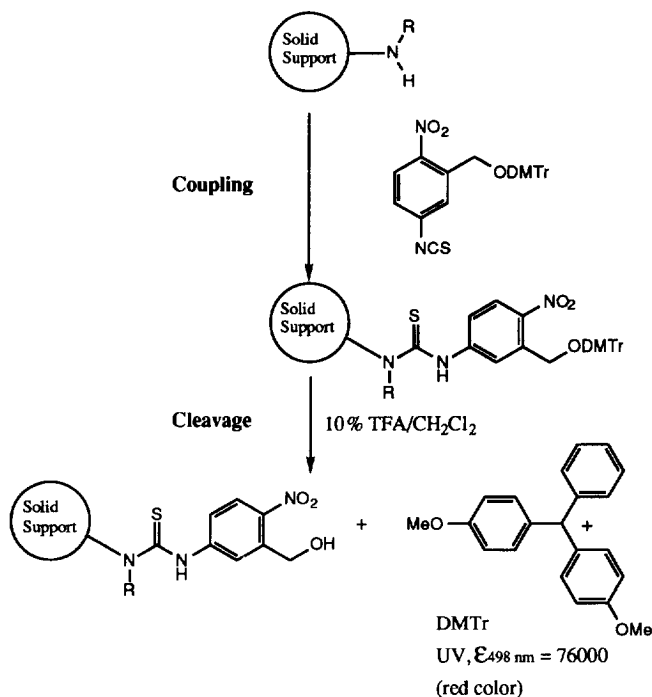
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**Abstract:** A new reagent, nitro phenylisothiocyanate-O-trityl (NPIT), has been developed for the quantitative or qualitative analysis of amines on solid support. NPIT can be used to efficiently monitor reactions involving less reactive amines during combinatorial synthesis of compound library mixtures.

The combinatorial synthesis of large collections of compounds has rapidly emerged as a powerful method for identifying biologically active molecules.<sup>1</sup> Significant advances have been made in the rapid synthesis of peptide and oligonucleotide mixtures and more recently in the synthesis of small organic non-peptide libraries on solid support.<sup>1b,2</sup> Verification of the fidelity of individual reactions in most cases has been accomplished by characterization of the final target compound. Methods for monitoring the extent of individual reactions quantitatively when *mixtures* of compounds are generated has received far less attention.<sup>3</sup> A number of methods have been reported for monitoring coupling reactions in conventional solid phase peptide synthesis where natural amino acids are used as the building blocks.<sup>4</sup> Typically the amount of unreacted amine remaining after a coupling cycle is determined through reaction with an amine-specific reagent. In the course of our work on non-peptide libraries we required a rapid, general, and quantitative method for monitoring coupling reactions of *non-natural* amino acids. In particular, we found that all existing methods for the detection of free amines (in natural amino acids) were unsatisfactory when applied to many of the nonbasic or relatively hindered amines used in our non-peptide library. Herein we describe the development and use of a new reagent, NPIT (nitro phenylisothiocyanate-O-trityl), which allows the efficient quantitative or qualitative analysis of less reactive amines on solid support during combinatorial synthesis.

The NPIT reagent consists of an activated isothiocyanate which selectively reacts with amines<sup>5</sup>, coupled to a trityl ether reporting group carrying a latent trityl cation which is released through mild acid hydrolysis.<sup>4b</sup> The amount of free amine is given by the concentration of trityl cation released which is readily measured in a UV spectrophotometer.<sup>6</sup> The procedure involves subjecting a small sample of dried resin (ca. 5 mg) to the simple protocol outlined in Figure 1.<sup>7</sup> If a qualitative assessment of the presence of free amine is

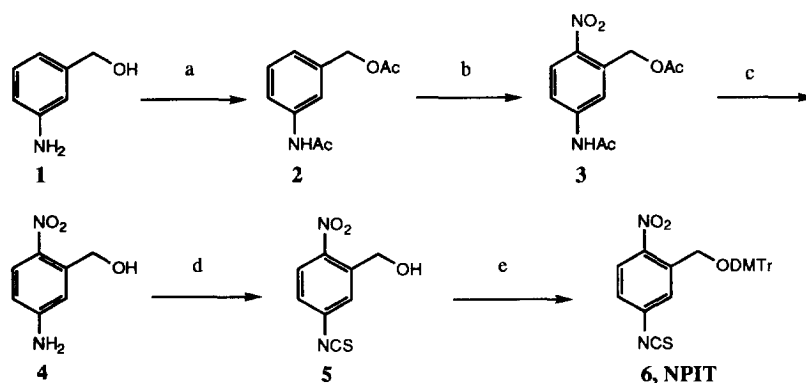


**Figure 1.** Reaction of NPIT reagent with free amines on solid support followed by release of dimethoxytrityl cation with TFA.

desired the resin, rinsed free of any excess reagent (wet), is briefly exposed to TFA vapors. The presence of residual free amine is assessed by the appearance of a distinct red color on the resin.

NPIT is a stable pale yellow solid, readily synthesized in five steps from commercially available 3-aminobenzyl alcohol as shown in Scheme 1. The aminobenzyl alcohol **1** is first protected as the diacetate followed by nitration to provide the desired 4-nitro isomer **3** in 40% yield. Deprotection followed by isothiocyanate generation and lastly dimethoxytrityl ether formation provides the NPIT reagent **6** in 10% overall yield. The NPIT reagent is an amorphous solid which is stable for months when stored at  $0^\circ\text{C}$ .

Comparison of the NPIT reagent with three other known methods for the detection of amines on solid support is shown in Table 1. The terminal amines selected for this comparison were particularly unreactive for steric reasons (**8**, **10** and **11**) or due to their weak basicity (**9**, anilinic). The terminal  $\beta$ -alanine **7** was included as a control. The DMTrCl reagent<sup>4b</sup> fails to react with hindered amine **11** resulting in a substitution level about 2% of theoretical. The picric acid test<sup>4c</sup> results in a substitution level only 80% of theoretical with aniline **9**,

Scheme 1<sup>a</sup>

<sup>a</sup>(a)  $\text{Ac}_2\text{O}/\text{Pyr}$ , 90%; (b)  $\text{HNO}_3/\text{H}_2\text{SO}_4$ , 40%; (c) 6 N  $\text{HCl}$ ,  $100^\circ\text{C}$ , 100%;

(d) Di-2-pyridyl thionocarbonate/ $\text{CH}_3\text{CN}$ , 73%; (e) 4,4'-Dimethoxytrityl chloride, DIEA, 40%.

presumably due to the weakly basic nature of this amine. More importantly, the picric acid test cannot discriminate between tertiary amines found in sidechains and the secondary or primary amine undergoing reaction, providing an overestimate in the substitution level with amine **10**. Lastly, the Kaiser test<sup>4a</sup> is not quantitative and yields inconclusive results with all the amines tested here except for the  $\beta$ -alanine control. Only the NPIT reagent consistently provides substitution levels which are within 3% of theoretical for all amines tested.

A reagent for monitoring reactions on solid support will preferably react quickly so that the analysis does not impede library synthesis. A kinetic study was undertaken to determine the rate of reaction of NPIT with the representative list of terminal amines (**7,9,10** and **11**, Figure 2) on solid support. NPIT reacts completely with all amines tested within 10 minutes. The importance of the nitro group in effectively activating the NPIT reagent is demonstrated by less than 50% reaction obtained with the unsubstituted reagent PIT after 40 minutes.

Lastly, we were interested in determining the sensitivity of the NPIT reagent, that is, what was the lower limit of free amine that could be detected with the reagent? An artificial mixture of amine **9** with 99 mol % tBoc protection and 1 mol % Fmoc protection was prepared on solid support. The Fmoc protecting group was selectively removed under standard conditions and the mixture containing minor free amine subjected to analysis with the NPIT reagent.<sup>8</sup> The residual amine detected using the NPIT reagent was 1.16%, in good agreement with the percentage of Fmoc protected amine in the original artificially generated mixture. The

ability to detect at least 1% free amine makes the NPIT reagent a sufficiently sensitive tool for monitoring chemistry involving primary or secondary amines on solid support.

(R = PAM- $\beta$ -Ala)

NC(=O)CCOP(=O)(O)O  
 $\beta$ -Ala (7)

NC1CCCC1C(=O)R  
 (8)

NC1=CC=C(C=C1)CC(=O)R  
 (9)

C1CCN(CC1)CC(=O)R  
 (10)

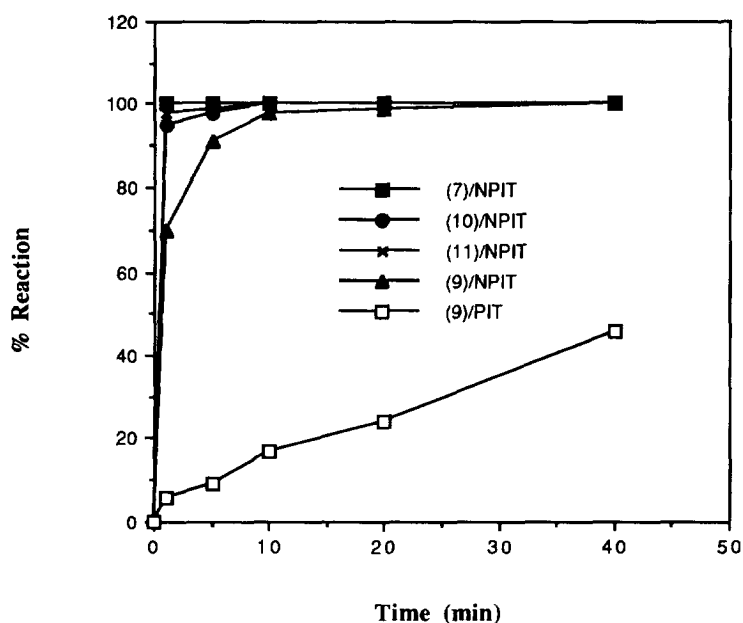
C1CCN(CC1)CC(=O)R  
 (11)

	$\beta$ -Ala (7)	(8)	(9)	(10)	(11)
NPIT	0.49 <sup>a</sup>	0.45 (0.46) <sup>b</sup>	0.45 (0.46)	0.46 (0.45)	0.47 (0.46)
DMTrCl	0.48	0.41 (0.45)	0.44 (0.45)	0.46 (0.44)	0.01 (0.45)
Picric Acid	0.53	0.50 (0.50)	0.40 (0.49)	0.70 (0.48)	0.48 (0.49)
Kaiser Test	dark blue <sup>c</sup>	dark brown	light brown	brown	brown

<sup>a</sup> Substitution level in mmol/g. <sup>b</sup> Number in parentheses is the theoretical value.

<sup>c</sup> Kaiser test only provides qualitative information based on colors observed.

**Table 1.** Methods for the determination of substitution level of amines on solid support.



**Figure 2.** Rate of reaction of NPIT with selected amines on solid support.

In conclusion, the NPIT reagent can be used to quantify a broad scope of free primary or secondary amines on solid support and therefore the extent of individual reactions in combinatorial synthesis. Free amines can be detected whether they exist as a homogeneous sample or if they consist of a mixture of different amines (e.g. when using pooling strategies<sup>9</sup>). The NPIT method is fast and sensitive and the reagent is a stable solid that is easy to prepare. It can also be used to screen out unreactive amines since an amine unable to react with NPIT would likely be too unreactive to participate in any coupling reaction for library synthesis.

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### References and Notes

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- (3) For more qualitative methods for monitoring reaction chemistry on solid support see: By C<sup>13</sup> NMR (a) Look, G.C.; Holmes, C.P.; Chinn, J.P.; Gallop, M.A. *J. Org. Chem.* **1994**, *59*, 7588 and references therein; this method requires isotopic labeling of individual monomers. By IR (b) see ref. 2c, also Frechet, J.M. Schuerch, C. *J. Am. Chem. Soc.* **1971**, *93*, 492.
- (4) (a) Kaiser Test: Kaiser, E. *Anal. Biochem.* **1970**, *34*, 595. Sarin, V.K.; Kent, S.B.H.; Tam, J.P.; Merrifield, R.B. *Anal. Biochem.* **1981**, *117*, 147. (b) 4,4'-Dimethoxytrityl chloride Method: Reddy, M.P.; Voelker, P.J. *Int. J. Pep. Prot. Res.* **1988**, *31*, 345. (c) Picric Acid Test: Gisin, B. *Anal. Chim. Acta.* **1972**, *58*, 248.
- (5) NPIT was found not to react with unprotected hydroxyl groups, i.e. no reaction was observed with an unsubstituted PAM resin.
- (6) It should be noted that use of the NPIT reagent, because it is a neutral molecule, produces a negligible background due to non-specific binding, in marked contrast with the dimethoxytrityl reagent (DMTrCl, ref. 4b) which can produce a significant background signal due to its charged nature.

- (7) The following protocol is used for reaction of NPIT with free amines on solid support:

Step	Operation	Volume (ml)	Time (min)
1	Couple with 0.1 M NPIT/DMF	0.5	1 x 10
2	Wash with DMF	1	2 x 1
3	Wash with CH <sub>2</sub> Cl <sub>2</sub>	1	3 x 1
4	Cleave with 2% TFA/CH <sub>2</sub> Cl <sub>2</sub>	1	3 x 2
5	Wash with CH <sub>2</sub> Cl <sub>2</sub>	1	5 x 1
6	Dilute cleavage and wash fraction of step 4 and 5 to 250 ml with 0.2% TFA/CH <sub>3</sub> CN		
7	Measure UV absorption at 498 nm		

A background measurement (for non-specific binding) can be made by first subjecting a separate sample of the resin to excess acetic anhydride to cap off any free amine prior to completing the above protocol.

- (8) This artificial mixture is representative of what would exist in a typical coupling reaction done on solid support where not all of the amine had reacted, that is, the 1% of free "unreacted" amine is evenly distributed on the solid support among the remaining protected amine (capped off).
- (9) We routinely use NPIT in small molecule combinatorial syntheses employing pooling methods and therefore large mixtures of different amines.

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