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Solid Phase Synthesis of Peptide *para* -Nitroanilides

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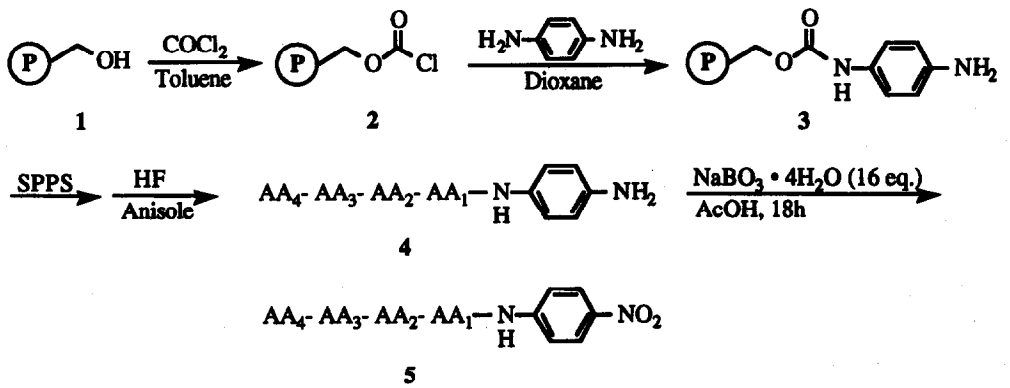
Abstract: A facile synthesis of peptide *para* -nitroanilides was developed using a novel urethane linked *para* -aminoanilide resin 3 and solid phase peptide synthesis. Conversion of the resulting peptide *para* -aminoanilides to the corresponding peptide *para* -nitroanilides was achieved by oxidation with sodium perborate. With the exception of sequences containing methionine, tryptophan and cysteine, all amino acid residues can be used. A representative synthesis of the tetrapeptide *para* -nitroanilide succinyl-Ala-Ala-Pro-Arg-PNA is described.

Peptide *para* -nitroanilide (PNA) chromogenic substrates have been used for many years to study the kinetics and specificity of proteolytic enzymes. However, these highly useful compounds are difficult to synthesize by classical solution methods.[1-8] We have devised a simple way to synthesize PNAs (Scheme 1) based upon an unique urethane linked *para* -aminoanilide (UPAA) resin **3** that is compatible with standard Merrifield solid phase peptide synthesis (SPPS) procedures.[9] Upon completion of the synthesis of the peptide, treatment with anhydrous HF yields the fully deprotected peptide *para* -aminoanilide (PAA) **4**. Oxidation of the PAA with sodium perborate tetrahydrate in glacial acetic acid generates the desired PNA **5**.[10]



Scheme 1

The speed and ease of SPPS allows a wide variety of N-terminal modifications of peptides but limits C-terminal modifications to free acids and amides. In the past, post synthetic modification or complete solution

synthesis of the peptide was necessary to generate the desired derivative. The simple synthesis and stability of the UPAA resin and the versatility of SPPS has allowed us to produce hundreds of PNAs for our collaborative research (11, 12). UPAA resin is compatible with all types of SPPS, and both Boc and Fmoc chemistries have been employed in our laboratory.This versatility allows novel amino acids and mixed chemistries to be used. Cleavage of the peptide from the resin produces the PAA, which is convieniently oxidized to the PNA with sodium perborate tetrahydrate.

Hydroxymethyl resin **1** with substitution levels of 1 mmol/g and lower can be purchased from a number of sources. However, to generate UPAA resin with > 1 mmol/g substitution, it is necessary to make hydroxymethyl resin from highly substituted chloromethyl resin. To 50 g of chloromethyl resin (1 mmol/g) was added 17.5 g (100 mmol) of N-Boc-glycine and 8.71 g (150 mmol) of potassium fluoride in 500 ml of N,N- dimethylformamide (DMF).[13] The mixture was heated to 80°C and stirred 12 hours to generate N-Boc-glycine resin. The resin was filtered and rinsed with DMF, water and MeOH. After drying the resin *in vacuo*, the substitution level of the resin was determined after deprotection by picrate titration.[14] The N-Boc-glycine resin was suspended in 500 ml of DMF and treated with 62.7 ml (2140 mmol) of hydrazine for 12 hours to yield hydroxymethyl resin. A small amount of resin was washed for 20 minutes with 50% trifluoro acetic acid (TFA) in dichloromethane (DCM), washed several times with DCM and then checked by the standard Kaiser test .[15] If the test was positive, the hydrazine procedure was repeated until the test was negative. The resin was then rinsed several times with toluene. Phosgene in toluene (40 ml, 160 mmol) was added to the resin 2 times for 20 minutes to generate the chloroformate intermediate **2**. After rinsing several times with toluene and dioxane, a saturated solution of 1,4-phenylenediamine in dioxane (100 ml) was added to the resin and stirred for 1 hour. After draining and rinsing with DMF, DCM and MeOH, the UPAA resin **3** was dried *in vacuo*, and the final substitution of 0.65 mmol/g was determined by picrate titration. Conversion to the TFA salt by washing with 50% trifluoro acetic acid (TFA) in dichloromethane (DCM) allows storage of the UPAA resin for up to 1 year at -20°C.

Synthesis of the peptide succinyl-Ala-Ala-Pro-Arg-PNA **7** was carried out using standard Boc SPPS protocols. Boc groups were cleaved for 20 minutes with a solution of consisting of 45% TFA, 45% DCM, 5% 1,2-ethanedithiol and 5% anisole, and the resin neutralized with a solution of 10% triethylamine in DCM. Boc amino acids were coupled with benzotriazol-1-ylosytris(dimethylamino)phosphonium hexafluorophosphate (BOP, 1.15 g, 2.6 mmol), 1- hydroxybenzotriazole (HOBt, 0.088 g, 0.65 mmol) and 4-methylmorpholine (NMM, 0.43 ml, 3.9 mmol). All couplings and deprotections were monitored using the Kaiser test. N-α-Boc-N-γ-p-tosyl-L-arginine (1.11 g, 2.6 mmol) was coupled to UPAA resin (1 g, 0.65 mmol) for 12 hours. The residues N-Boc-L-proline (0.55 g, 2.6 mmol), N-Boc-L-alanine (0.49 g, 2.6 mmol) and N-Boc-L-alanine (0.49 g, 2.6 mmol) were coupled sequentially using the same protocol except that the coupling time was 1 hour. Final succinylation was performed using succinic anhydride (0.26 g, 2.6 mmol) in DCM for 15 minutes.

The peptide resin was dried *in vacuo*, and the peptide cleaved from the resin with 20 ml anhydrous hydrofluoric acid containing 2 ml of anisole at 0°C for 1 hour. After removal of the HF, the resin was washed extensively with diethyl ether. The fully deprotected peptide was extracted away from the resin with glacial acetic acid and diluted to a theoretical concentration of 5 mg/ml. The PAA **6** was then converted to the PNA **7**

in the presence of 16 eq of sodium perborate tetrahydrate for 18 hours at room temperature. The acetic acid was removed *in vacuo* resulting in an orange/brown solid.

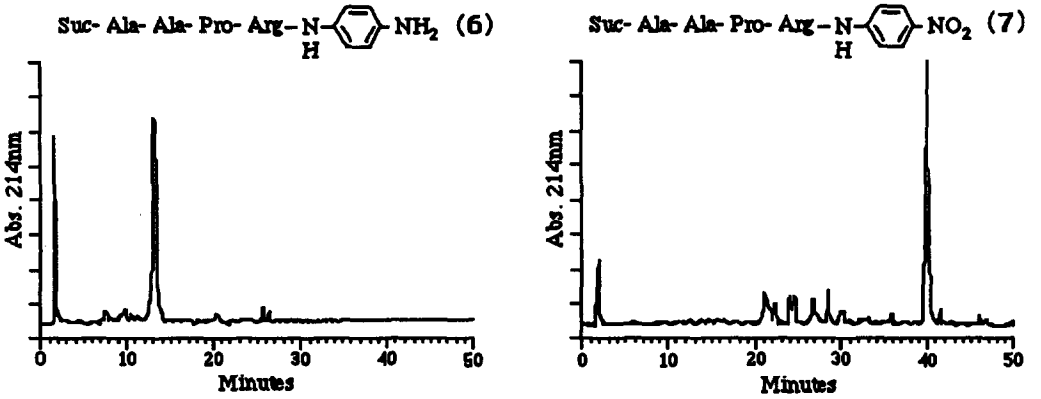


Figure 1: Comparison of the crude PAA (6) and PNA (7). Analytical HPLC conditions were 0.5 % acetonitrile/minute gradient, UV at 214 nm, flow rate of 2 ml/minute, 4.6 mm x 250 mm 5μ 300Å Vydac C_{18} column.

The PNA salt was then purified by reverse phase HPLC using a gradient of 0.175 percent per minute acetonitrile 0.1% TFA/water 0.1% TFA over a 2.5 cm x 25 cm, 15-20 μ 300 Å Vydac C_{18} column at a flow rate of 18 ml per minute yielding 128 mg of pure peptide. Figure 1 shows a comparison of the crude peptides succinyl-Ala-Ala-Pro-Arg-PAA and succinyl-Ala-Ala-Pro-Arg-PNA. The PAA elutes at 13 minutes or 6.5% acetonitrile/water while the PNA elutes much later at 40 minutes or 20% acetonitrile/water. This longer retention aided the purification because the salts and excess acetic acid associated with the peptide elute well before the PNA does. In a preparative run, the salts and acetic acid create a large flow through peak in the profile. When producing large quantities of PNA (>1 gm), we have found it highly advantageous to perform a desalting step on the crude peptide before introducing it to our purification column.[16]

We have found that our method of synthesizing peptide para-nitroanilide substrates works for most amino acid sequences except except those containing easily oxidizable amino acids such as methionine, tryptophan or cysteine. In the past years we have produced hundreds of tri- and tetrapeptide PNAs, often in gram quantities, for use in our collaborative research.

References and Notes

1) Sharma, S.K. and Castellino, F.J. *Thrombosis Res.* **1990**, *57*, 127-138.

2) Okada, Y., Tsuda, Y., Nagamatsu, Y. and Okamoto, U. *Int. J. Peptide Protein Res.* **1981**, *17*, 560-564.

3) Okada, Y., Tsuda, Y., Nagamatsu, Y. Tęno, N., Wanaka, K., Sasaki, K., Hijikata, A., Naito, T. and Okamoto, S. *Int. J. Peptide Protein Res.* **1986**, *27*, 79-85.

4) Cs.-Szabo, G., Pozsgay, M. and Elodi, P. *Thrombosis Res.* **1980**, *20*, 199-206.

5) Erlanger, B.F., Kokowsky, N. and Cohen, W. *Arch. Biochem. Biophys.* **1961**, *95*, 271-278.

6) Somorin, O. Nishi, N. and Noguchi, J. *Bull. Chem. Soc. Jap.* **1978**, *51*, 1255-1256.

7) Roffman, S. and Troll, W. *Anal. Biochem.* **1974**, *61*, 1-5.

8) DelMar, E.G., Largman, C., Brodrick, J.W. and Geokas, M.C. *Anal. Biochem* **1979**, *99*, 316-320.

9) Merrifield, R. B. J. Am. Chem. Soc. **1963**, *85*, 2149-2154.

10) McKillop, A. and Tarbin, J.A. *Tetrahedron Lett.* **1983**, *24*, 1505-1508.

11) Bott, R., Ultsch, M.,Wells, J., Powers, D., Burdick, D., Struble, M., Burnier, J., Estell, D., Miller, J., Graycar, T., Adams, R., Power, S. *Biotechnology in Agricultural Chemistry, ACS Symposium Series No. 334*, **1987**, 139-147.

12) Estell, D. A., Graycar, T.P., Miller, J.V., Powers, D.B., Burnier, J.P., Ng, P.G., Wells, J.A. *Science* **1986**, *233*, 659-663.

13) Horiki, K., Igano K. and Inouye, K. *Chem. Lett.* **1978**, 165-168.

14) Gisin, B. *Anal. Chim. Acta* **1972**, *58*, 248-249.

15) Kaiser, E., Colescott, R.L., Bossinger, C.D., and Cook, P.I. *Anal. Biochem.* **1970**, *34*, 595-598.

16) 3 to 5 grams (theoretical) of PNA was dissolved in 6 liters of 10% acetic acid, 10% acetonitrile in water and pumped onto a preparative reverse phase column. 3 column volumes of water were passed over the column to remove all of the acetic acid and salt leaving the PNA bound to the column. Then 60% acetonitrile/water was passed over the column to elute the PNA in a small volume. This material was collected, concentrated and then fractionated using preparative HPLC.

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