Reinvestigation of the Phosphazo Method and Synthesis of *N*-(*t*-Butoxycarbonyl)-L-arginine *p*-Nitroanilide and a Chromogenic Enzyme Substrate for the Factor Xa

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Synopsis. Reaction conditions for the phosphazo method were reinvestigated in order to apply this method to the synthesis of *p*-nitroanilide(*p*NA)s of *t*-butoxycarbonyl(Boc)- and benzyloxycarbonyl(Z)-amino acids.

p-Nitroanilides of amino acids and peptides are among the most popular chromogenic enzyme substrates and their synthesis has been studied intensively. However, since *p*-nitroaniline is a very poor nucleophile, the usual peptide bond forming reactions including the dicyclohexylcarbodiimide-*N*-hydroxybenzotriazole method[1] and the mixed anhydride method[2] could not give sufficient results. As an alternative, the reaction of N^{α}-protected amino acids with *p*-nitrophenyl isocyanate, isolated[3] or obtained in situ by the modified Curtius reaction using diphenyl phosphorazidate,[4] has up until now been the method of choice. The phosphazo method,[5] which had fallen into disuse as a general method for peptide synthesis, was recently utilized for the synthesis of *p*-nitroanilides.[1] However, its applicability to Boc-amino acids had not been examined. In this study we reinvestigated and optimized the conditions for the formation and reaction of the phosphazo compound from *p*-nitroaniline in order to establish a synthetic method for Boc-amino acid *p*-nitroanilides including Boc-arginine *p*-nitroanilide.

Results and Discussion

Optimization of the conditions of the phosphazo method was achieved by focusing on the following points; 1) the reaction time necessary for the complete formation of the phosphazo derivative of *p*-nitroaniline, 2) the lowest necessary temperature for the reaction of the phosphazo compound with acyl components, and 3) molar ratios.

p-Nitroaniline (**1**) was mixed with phosphorus trichloride (**2**) in pyridine at room temperature for 1 h to afford the corresponding phosphazo compound, *N,N'*-bis(4-nitrophenyl)phospheneimidous amide (**3**). To this *Z*-L-Phe-OH (**4**) was added (molar ratio of **2**:**4**=1:1) and the mixture stirred for 30 min. Then the temperature was elevated stepwisely to 30, 40, 50, and finally to 60 °C maintaining each temperature for 30 min. At the end of each period the amount of *Z*-L-Phe-*p*NA (**5**) formed was quantitated by HPLC. The yield dramatically increased (24→84%) when the temperature was elevated from 30 °C to 40 °C. Therefore, it was concluded that the lowest necessary temperature for the reaction of **3** with the acyl component was 40 °C. This temperature is much lower than the boiling point of pyridine which had been previously used.[5]

In the above reaction the first step (the reaction of **1** with **2**) was carried out for 1 h. When this step was interrupted after 15 min, as described in the literature,[5] by the addition of **4** and warming the mixture to 40 °C, the reaction was not complete after 4 h. On the other hand, when the first step was continued for 1 h, subsequent reaction with **4** was complete after 1 h. Although this reaction time was not the minimal time required, it was used in the subsequent reactions.

The molar ratio of **2** to **4** appears to be another important factor. In the phosphazo method carboxyl components are usually used in excess, however yields of *p*-nitroanilides under these conditions are low. Better results were obtained when the phosphazo derivative of *p*-nitroaniline was used in excess. When 1.2 and 2.0 equivalents of the phosphazo compound (molar ratios of **2**:**4**=0.6:1 and 1:1, assuming the complete formation of the phosphazo compound) were used yields of the *p*-nitroanilide **5** after 1 h were 81% and quantitative, respectively. Since the former yield could not be significantly improved by increasing the reaction time, it was concluded that an equimolar amount of the phosphazo compound, which corresponds to 2 equivalents of the acyl component, should be used.

In summarizing the above results the optimal conditions for the synthesis of protected amino acid *p*-nitroanilides were decided as follows: 2 mol of *p*-nitroaniline are mixed with 1 mol of PCl_3 in pyridine for 1 h at room temperature. After the addition of 1 mol of a protected amino acid to the mixture the reaction is performed at 40 °C for 1 h or more depending on the structure of the amino acid used.

Under these conditions syntheses of various kinds of N^{α}-protected amino acid *p*-nitroanilides were performed and the results are summarized in Table 1. As the result of the lowering of the reaction temperature, preparation of Boc- and *p*-methoxybenzyloxycarbonyl-amino acid *p*-nitroanilides was possible (Entries 6, 7, 8, 9, and 10). It should also be noted that dicyclohexylamine (DCHA) salts of protected amino acids could be used quite as same as the free acids. Using this technique *p*-nitroanilide of Boc-L-glutamine was obtained, albeit in modest yield because of nitrile formation.[10]

N^{α}-Acylated arginine *p*-nitroanilides are well-known as substrate for trypsin,[11] papain,[12] tissue plasminogen activator, and plasmin.[13] In the synthesis of these compounds the guanidino function of arginine was protected by nitro[11,13] or arenesulfonyl groups.[12] However strong acid treatment in the total deprotection step often gives a complex mixture of

Table 1. Preparation of Protected Amino Acid *p*-Nitroanilides

Entry	Product (Method[a])	Reaction time/h	Yield %	Mp/°C (solvent)[b]	Elemental analysis	$[\alpha]_D$/degree (c 1, CH_3OH)	R_f[c] A	B	C	D
1	Z-L-Phe-*p*NA(A)	1.5	91	158—160(A)[d]		+48.4(25 °C)	0.23	0.91		
2	Z-L-Val-*p*NA(A)	3.0	73	188—191(A)	$(C_{19}H_{21}N_3O_5)$C,H,N	−14.8(27 °C)[e]	0.17	0.85		
3	Z-L-Leu-*p*NA(A)	1.5	79	128—129(A)[f]	$(C_{20}H_{23}N_3O_5)$C,H,N	−21.1(28 °C)	0.22	0.89		
4	Z-L-Ile-*p*NA(A)	4.0	49	163—164(B)	$(C_{20}H_{23}N_3O_5)$C,H,N	−10.6(28 °C)	0.20	0.89		
5	Z-L-Arg-*p*NA·HCl(A)	4.0	94	176—179[g]	$(C_{20}H_{25}N_6O_5Cl)$C,H,N	−12.0(28 °C)			0.39	0.52
6	Z(OMe)-L-Leu-*p*NA(B)	1.5	74	107—108(A)	$(C_{21}H_{25}N_3O_6)$C,H,N	−11.9(29 °C)	0.19	0.90		
7	Boc-L-Leu-*p*NA(A)	1.5	89	123—125(C)[h]	$(C_{17}H_{25}N_3O_5)$C,H,N	−28.5(29 °C)	0.26	0.91		
8	Boc-L-Leu-*p*NA(B)	1.5	87	121—123(C)[h]		−30.0(26 °C)	0.26	0.93		
9	Boc-L-Lys(Z)-*p*NA(A)	2.0	60	Amorphous[i]	$(C_{25}H_{32}N_4O_7)$C,H,N	−7.6(30 °C)	0.10	0.83		
10	Boc-L-Gln-*p*NA(B)	2.0	43	166—168(decomp)(A)	$(C_{16}H_{22}N_4O_6)$C,H,N	−14.0(28 °C)		0.34		0.84
11	Boc-L-Arg-*p*NA·HCl(A)	4.0	75	195—198(decomp)(D)[j]	$(C_{17}H_{27}N_6O_5Cl)$C,H,N	−9.6(29 °C)[e]			0.31	0.51

a) See Experimental. b) A: ethyl acetate–petroleum ether; B: ethyl acetate–ether–petroleum ether; C: ether–petroleum ether; D: ethanol. c) A: chloroform; B: chloroform : methanol=9 : 1; C: chloroform : methanol : acetic acid=20 : 5 : 1. d) Lit,[6] mp 158.5—159.5 °C. e) (c 0.5, CH_3OH). f) Lit,[7] mp 148—151 °C. g) Lit,[8] mp 181—182 °C, $[\alpha]_D^{20}$—16.0° (c 1, EtOH). h) Lit,[4] mp 91—96 °C, $[\alpha]_D^{20}$—30.4° (c 1, CH_3OH); Lit,[2] mp 80—81 °C, $[\alpha]_D^{20}$—27.5° (c 1, CH_3OH). i) Lit,[4] mp 113—114 °C, $[\alpha]_D^{20}$—8.3° (c 1, CH_3OH). j) Lit,[9] mp 190—192 °C.

products. The most important feature of the modified phosphazo method is the facile availability of Boc-L-Arg-*p*NA, which was obtained as a crystalline hydrochloride (**6**) in 75% yield starting from Boc-L-Arg-OH·HCl·H_2O. To the best of our knowledge, synthesis of Boc-L-Arg-*p*NA by direct coupling of Boc-arginine and *p*-nitroaniline with phosphorus trichloride in pyridine has been recorded only in a patent.[9]

Compound **6** thus obtained was then utilized for the preparation of a peptide *p*-nitroanilide, Bz-L-Ile-L-Glu(OMe)-Gly-L-Arg-*p*NA (**7**), which is known as a substrate for the factor Xa.[14] Protected *N*-terminal tripeptide ester, Boc-L-Ile-L-Glu(OMe)-Gly-OPac, was obtained by stepwise elongation by the mixed anhydride method using dimethylphosphinothioyl chloride.[15] After the Boc group was changed to benzoyl group, the C-terminal phenacyl ester was removed to give protected tripeptide acid, Bz-L-Ile-L-Glu(OMe)-Gly-OH (**8**). Segment condensation of **8** with arginine *p*-nitroanilide dihydrochloride (**9**), which was obtained by deprotection of **6** with HCl in acetic acid, was performed using dimethylphosphinothioyl azide[16] in *N*,*N*-dimethylformamide. Since the final deprotection step was not necessary in this synthesis, the pure product was readily obtained.

Experimental

Thin-layer chromatography was carried out on silica-gel plates (Merck, Kieselgel 60 F_{254}) in the following solvent systems; A: $CHCl_3$, B: $CHCl_3$: CH_3OH=9 : 1, C: $CHCl_3$: CH_3OH=4 : 1, D: $CHCl_3$: CH_3OH : CH_3CO_2H=20 : 5 : 1. For amino acid analysis peptides were hydrolyzed with constant boiling hydrochloric acid in evacuated and sealed ampoules for 24 h at 110 °C. The analyses were performed on an Atto Model 203 Amino Acid Analyzer. Optical rotations were determined using a JASCO DIP-360 digital polarimeter. ^1H NMR spectra were observed with a Hitachi R-1100 and JEOL JNM-PMX-60 spectrometers (60 MHz). Analytical reversed-phase HPLC was performed using a Unisil Q C_{18} column (4.6×300 mm) with an isocratic solvent system (0.01 M HCl : CH_3OH=35 : 65, flow rate 1.0 ml min^{-1}) at 60 °C using Shimadzu LC 6A liquid chromatograph system monitored at 254 nm (1 M= 1 mol dm^{-3}).

General Procedure for the Preparation of Protected Amino Acid *p*-Nitroanilides: To a solution of phosphorus trichloride (0.872 ml, 10 mmol) in pyridine (10 ml) was added *p*-nitroaniline (1.38 g, 20 mmol) at 25 °C, and the mixture stirred for 1 h. To this solution was added a protected amino acid (Method A) or a protected amino acid DCHA salt (Method B) (10 mmol) in pyridine (20 ml) and the mixture stirred at 40 °C for the period given in Table 1. After removal of the solvent under reduced pressure the residue was triturated with ethyl acetate and water and filtered. The organic layer was separated and washed successively with a 5% citric acid solution (3 times), water, a 5% sodium hydrogencarbonate solution (3 times), water and saline, and dried over sodium sulfate. After removal of the solvent, the crystalline residue was rinsed with cold benzene or chloroform and then with ether before recrystallization. When necessary silica-gel column chromatography using dichloromethane for elution was performed prior to recrystallization.

***N*-(*t*-Butoxycarbonyl)-L-arginine *p*-Nitroanilide Hydrochloride (6):** To a solution of the phosphazo compound prepared from PCl_3 (0.436 ml, 5 mmol) and *p*-nitroaniline (1.381 g, 10

mmol) was added Boc-L-Arg-OH·HCl·H_2O (1.644 g, 5 mmol) in pyridine (5 ml). The mixture was stirred at 40 °C for 4 h and the solvent removed under reduced pressure. The residue was dissolved in water (50 ml) and ether (20 ml) and kept at 0 °C overnight. Precipitates were collected by filtration, washed successively with ether, dichloromethane and a small quantity of ethanol and recrystallized from ethanol-ether to give **6** as a colorless granular solid; 1.616 g (75%); ^{1}H NMR ($CDCl_3$+DMSO-d_6) δ=1.4 (s, 9H), 1.5—1.9 (m, 6H), 3.1—3.4 (m, 2H), 4.25 (br, 1H), 6.7—7.3 (m, 4H), 7.6—8.2 (m, 5H), 10.9 (s, 1H).

***N*-(*t*-Butoxycarbonyl)-L-glutamine *p*-Nitroanilide and *N*-(*t*-Butoxycarbonyl)-(2-cyanoethyl)glycine *p*-Nitroanilide:** Boc-L-Gln-OH·DCHA (4.276 g, 10 mmol) was treated with the phosphazo compound as described above. After the usual washing procedures, the products were separated by silica-gel column chromatography using dichloromethane-methanol (50 : 1 v/v) as eluent to give 1.578 g (43%) of Boc-L-Gln-*p*NA as colorless crystals; ^{1}H NMR ($CDCl_3$+DMSO-d_6) δ=1.4 (s, 9H), 1.8—2.6 (m, 4H), 4.1—4.4 (br, 1H), 6.0—6.4 (br, 2H), 6.8—7.0 (br, 2H), 7.7 (d, 2H), 8.1 (d, 2H), 10.5 (br, 1H). Further elution gave 0.802 g (23%) of Boc-(2-cyanoethyl)glycine-*p*-nitroanilide as a fluffy solid; mp 148—149 °C; $[\alpha]_D^{27}$ −21.1° (*c* 1.1, CH_3OH); IR (KBr) 2260 cm^{-1} (C≡N); ^{1}H NMR ($CDCl_3$) δ=1.4 (s, 9H), 1.9—2.7 (m, 4H), 4.3—4.7 (br, 1H), 5.5—6.9 (br, 1H), 7.6 (d, 2H), 8.0 (d, 2H), 9.2—9.5 (br, 1H).

L-Arginine *p*-Nitroanilide Dihydrochloride (9): To a solution of **6** (1.077 g, 2.5 mmol) in acetic acid (2 ml) was added 2.17 M HCl in acetic acid (23 ml) at RT. The mixture was kept standing for 2 h and diluted with ether. Precipitates were collected by filtration, washed with ether and dried to give **9** as a pale yellow powder: 883 mg (96%); mp 262—264 °C (decomp); $[\alpha]_D^{26}$ +73.2° (*c* 1, 0.1 M HCl). Lit,[8] mp 245 °C; $[\alpha]_D^{20}$ +80.6° (*c* 1, water). Found: C, 39.44; H, 5.55; N, 22.65%. Calcd for $C_{12}H_{18}N_6O_3$·2HCl: C, 39.25; H, 5.49; N, 22.88%.

***N*-Benzoyl-L-isoleucyl-L-α-glutamylglycyl-*N*-(4-nitrophenyl)-L-argininamide Methyl Ester [Bz-L-Ile-L-Glu(OMe)-Gly-L-Arg-*p*NA] (7):** To an ice-cooled solution of Bz-L-Ile-L-Glu(OMe)-Gly-OH (0.218 g, 0.5 mmol), **9** (0.184 g, 0.5 mmol) and triethylamine (70 μl, 0.5 mmol) in *N*,*N*-dimethylformamide (1 ml) was added dimethylphosphinothioyl azide (0.081 g, 0.6 mmol) in *N*,*N*-dimethylformamide (0.5 ml) and triethylamine (70 μl, 0.5 mmol). The mixture was stirred for 24 h at 0 °C and concentrated. The residue was solidified by trituration with ether and cold 1 M hydrochloric acid and purified by preparative thin-layer chromatography ($CHCl_3$: CH_3OH : CH_3CO_2H=20 : 5 : 1) and gel chromatography on Sephadex LH-20 (CH_3OH) to give 224 mg (58%) of **7** as an amorphous solid: $[\alpha]_D^{26}$ −31.3° (*c* 0.5, CH_3OH); R_{f_4}=0.51. Amino Acid Ratios: Ile, 0.96; Glu, 1.01; Gly, 1.00; Arg, 1.04. Found: C, 51.76; H, 6.26; 16.65%. Calcd for $C_{33}H_{45}O_9N_9$·HCl·H_2O: C, 51.73; H, 6.31; N, 16.45%.

References

Abbreviations according to IUPAC-IUB Commision, *Eur. J. Biochem.*, **138**, 9 (1984), are used throughout. Additional abbreviations in addition to those shown in the text: Ac, acetyl; Bz, benzoyl; OPac, phenacyl ester; *p*NA, *p*-nitroanilide; TEA, triethylamine; Z(OMe), *p*-methoxybenzyloxycarbonyl.

1) Y. Okada, Y. Tsuda, A. Hirata, Y. Nagamatsu, and U. Okamoto, *Chem. Pharm. Bull.*, **30**, 4060 (1982).

2) K. Noda, M. Oda, M. Sato, and N. Yoshida, *Int. J. Peptide Protein Res.*, **36**, 197 (1990).

3) N. Nishi, S. Tokura, and J. Noguchi, *Bull. Chem. Soc. Jpn.*, **43**, 2900 (1970).

4) T. Shioiri, M. Murata, and Y. Hamada, *Chem. Pharm. Bull.*, **35**, 2698 (1987).

5) S. Goldschmidt and G. Rosculet, *Chem. Ber.*, **93**, 2387 (1960).

6) E. V. Ramenskii, M. M. Botvinik, and R. U. Beisembaeva, *Khim. Prir. Soedin.*, **4**, 23 (1968); *Chem. Abstr.*, **69**, 87424n (1968).

7) H. Tuppy, U. Wiesbauer, and E. Wintersberger, *Hoppe-Seyler's Z. Physiol. Chem.*, **329**, 278 (1962).

8) O. Somorin, N. Nishi, and J. Noguchi, *Bull. Chem. Soc. Jpn.*, **51**, 1255 (1978).

9) S. Bajusz, A. Juhase, E. Barabas, D. Bagdi, and L. Mohai, *Hung. Teljes* HU 40615; *Chem. Abstr.*, **108**, 112956w (1988).

10) D. T. Gish, P. G. Katsoyannis, G. P. Hess, and R. T. Stedman, *J. Am. Chem. Soc.*, **78**, 5954 (1956); C. Ressler, *ibid.*, **78**, 5956 (1956).

11) S. Tokura, N. Nishi, and J. Noguchi, *J. Biochem.*, **69**, 599 (1971).

12) H. Nakata and S. Ishii, *Biochem. Biophys. Res. Commun.*, **41**, 393(1970).

13) S. K. Sharma and F. J. Castellino, *Thromb. Res.*, **57**, 127 (1990).

14) L. Aurell, R. Simonsson, S. Arielly, G. Karlsson, P. Friberger, and G. Claeson, *Haemostasis*, **7**, 92 (1978).

15) M. Ueki and T. Inazu, *Chem. Lett.*, **1962**, 45.

16) M. Ueki, K. Okazaki, and S. Ikeda, "Peptide Chemistry 1984: Proc. of the 22nd Symposium on Peptide Chemistry," ed by N. Izumiya, Protein Research Foundation, Osaka (1984), p. 67.