$CHCl_3$ gave a single peak on GLC. MS *m/e* 444 (M^+), 426 (M^+-H_2O), 273 ($M^+-C_{10}H_{19}O_2$), 255, 137, 119, 74 ($C_3H_6O_2$), 73 ($C_3H_5O_2$) and 44 (CO_2). High resolution MS, Calcd for $C_{29}H_{48}O_3$ (M^+): 444.3603. Found: 444.3600.

Acknowledgement The authors are grateful to Dr. N. Ikekawa for interpretation of ^{13}C-NMR spectra. They are also grateful to Emeritus Prof. K. Hisauchi and Prof. M. Ikuse of this faculty for encouragement throughout this work. Thanks are also due to Miss. Y. Sakamoto of this faculty for PMR and ^{13}C-NMR measurements, to Dr. T. Morikawa for elemental analysis, and to Mr. M. Takayama for determining MS spectra.

[Chem. Pharm. Bull. 28(6)1899—1905(1980)]

Inverse Substrates. IX.[1] Amidinophenyl Esters derived from Amino Acids and Peptides: Synthesis and Properties as Trypsin Substrates

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(Received December 13, 1979)

Methods for the preparation of *p*-amidinophenyl esters from amino acid derivatives are described. Blocking of the amidino function with the benzyloxycarbonyl group and its deblocking by catalytic hydrogenation after coupling were satisfactory. *p*-Amidinophenyl esters are of special interest because of their susceptibility to the hydrolytic enzyme, trypsin, and some parameters of the hydrolysis of these compounds by trypsin are presented.

Keywords——*p*-amidinophenyl ester; synthetic substrates; ester formation; trypsin; enzyme kinetics; deacylation rate constant

In our previous papers,[3] it was shown that esters of *p*-amidinophenol are specifically hydrolyzed by trypsin. These esters are characterized by their linkage, *i.e.*, the site-specific group (charged amidinium) for the enzyme is not located in the acyl moiety but in the leaving portion. A new term, "inverse substrates", was proposed for these esters, having regard to their specific binding and efficient acylation processes, comparable to those for normal-type substrates.[3a] This has provided, for the first time, a general method for the *specific* introduction of an acyl group carrying a *non-specific* residue into the trypsin active site without recourse to a cationic acyl moiety characteristic of conventional substrates.[3d] It is of interest to investigate the behavior of trypsin towards "inverse substrates" derived from a variety of amino acids and preptides, including amino acids of the D-series. Analysis of the deacylation process in the trypsin-catalyzed hydrolysis of this entirely new type of substrates could shed light on functions of the enzyme which are not manifested with typical substrates. This report is mainly concerned with the synthesis of these "inverse substrates" from various amino acids and some peptides. Their behavior towards trypsin is also described briefly.

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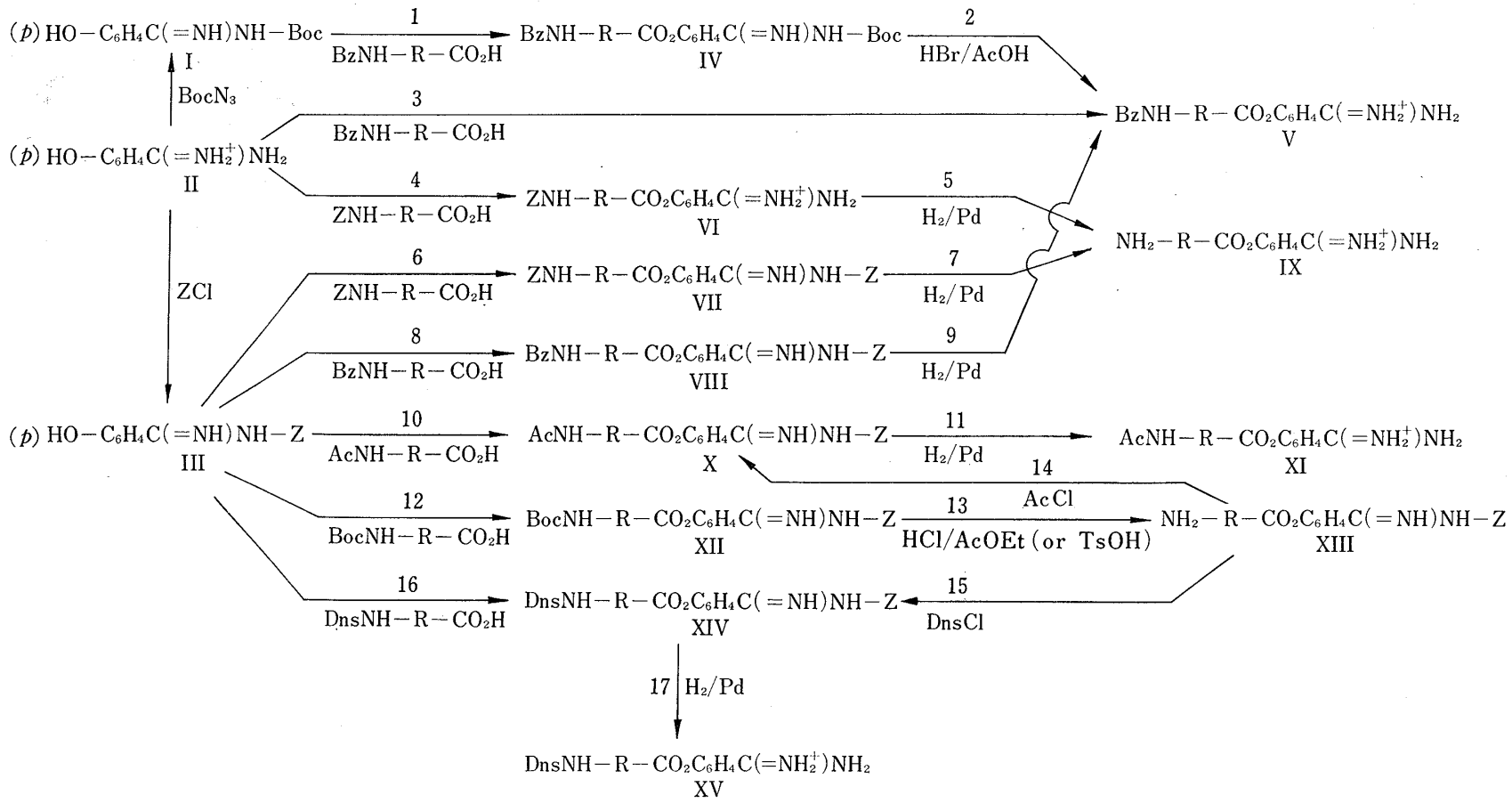

TsOH; *p*-toluenesulfonic acid, Boc; *t*-butyloxycarbonyl, Z; benzyloxycarbonyl, Bz; benzoyl, Dns; 1-dimethylamino-5-naphthalenesulfonyl, Ac; acetyl, NH–R–CO; amino acid or peptide residue

Chart 1

Synthetic routes to "inverse substrates" are shown in Chart 1. Except in a few cases, N-blocked amidinophenols were used for the coupling reaction. The low solubility of *p*-amidinophenol in organic solvents was often a serious problem in synthesizing the esters. Thus, the N-benzyloxycarbonyl (or *t*-butyloxycarbonyl) derivative not only prevents possible N-acylation reactions but is superior in solubility and reactivity, and is preferable to the unsubstituted *p*-amidinophenol. The *p*-amidino group, with a Hammett σ value of $+0.65$,[4] has a strong electronwithdrawing effect on the phenol hydroxyl group. Because of this poor nucleophilic character of *p*-amidinophenol derivatives, common procedures for the coupling reaction of carboxylic acids and phenols were not applicable. Each ester was obtained only after a number of trials with a variety of dehydrating reagents. Optically active amino acid derivatives (acetyl) were prepared through the esters of *t*-butyloxycarbonylamino acids, followed by removal of the *t*-butyloxycarbonyl group and acetylation as shown in Chart 1. Deblocking of the benzyloxycarbonyl group at the amidine function was successfully carried out by catalytic hydrogenation. Treatment with hydrogen bromide in acetic acid was ineffective, in contrast to the case of the amino group.

Trypsin-catalyzed hydrolysis of "inverse substrates" was monitored spectrophotometrically, using the procedure described previously.[3a] The rates of the rapid acylation of trypsin were determined by the stopped flow technique. The subsequent deacylation process is usually slow. As a representative example, the time course of tryptic hydrolysis of esters of acetylalanine is shown in Fig. 1. Kinetic parameters for these esters were as follows: K_s, 46 μM; k_2, 4.8 s^{-1}; k_3, 2.1 s^{-1} for the L-series and K_s, 30 μM; k_2, 7.0 s^{-1}; k_3, 0.01 s^{-1} for the D-series. Comparison of the deacylation rate constants, k_3, in the enantiometric pair is of particular value in analyzing the structure-function relationship of the enzyme. Such a parameter for D-amino acids could not previously be obtained because conventional esters of D-amino acids are never involved in the enzymatic process. The behavior of this series of compounds with trypsin will be reported in detail in a subsequent paper.

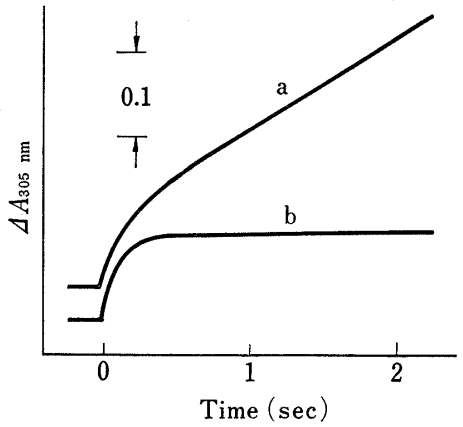

Fig. 1. Trypsin-catalyzed Hydrolysis of "Inverse Substrates" at pH 8.0, 25°

a: N-acetyl-L-alanine *p*-amidinophenyl ester.
b: N-acetyl-D-alanine *p*-amidinophenyl ester.
$[E]_0$: 1.83×10^{-6} M, $[S]_0$: 7.4×10^{-5} M.

Experimental

p-Amidinophenyl esters were prepared by the routes shown in Chart 1, and their physical data are summarized in Table I. Table II lists the data for the intermediates, N-benzyloxycarbonyl-*p*-amidinophenyl esters and N-*t*-butyloxycarbonyl-*p*-amidinophenyl ester.

N-(*t*-Butyloxycarbonyl)-*p*-amidinophenol (I)——*t*-Butyl azideformate (5.7 g, 0.046 mol) was added dropwise with stirring to a solution of *p*-amidinophenol (II) hydrochloride (3.5 g, 0.02 mol) and triethylamine (6.1 g, 0.06 mol) in 40 ml dimethylformamide (DMF), at room temperature. After standing at room temperature for 72 hr, water (200 ml) was added to the reaction mixture, and the resulting precipitate was filtered off. The precipitate, which contained both N-blocked and N,O-di-blocked amidinophenols, was subjected to hydrolysis. After dissolving it in ethanol (80 ml), 5% NaOH solution was added dropwise and the solution was kept at room temperature for 1.5 hr. The solution was then adjusted carefully to pH 7 with dil. HCl. The resulting precipitate was collected and dried in a desiccator over NaOH pellets. Recrystallization from ethyl acetate gave colorless prisms, 4.2 g (90%), mp 183—185°, *Anal.* Calcd for $C_{12}H_{16}N_2O_3$: C, 61.00; H, 6.83; N, 11.86. Found: C, 60.82; H, 6.70; N, 11.66.

N-(Benzyloxycarbonyl)-*p*-amidinophenol (III)——Benzyloxycarbonyl chloride (17 g, 0.1 mol) was added dropwise at 0° to a solution of II (hydrochloride) (5.1 g, 0.03 mol) and triethylamine (14 g, 0.14 mol) in DMF

4) C.-H. Wang and E. Shaw, *Arch. Biochem. Biophys.*, **150**, 259 (1972).

Table I. Synthetic Routes and Physical Data for *p*-Amidinophenyl Esters

Compd. No.	Compound (*p*-Toluenesulfonate)	Synthetic[b] pathway	Total yield (%)	mp (°C, dec.) (from)	Formula	Analysis (%) Calcd (Found) C	H	N	S	$[\alpha]_D^{20}$ (c=1.0, MeOH)
Va	N-Benzoyl-β-alanine *p*-amidinophenyl ester	3	72	184—186.5 (EtOH–CH_3CN)	$C_{24}H_{25}N_3O_6S$	59.61 (59.23	5.21 5.20	8.69 8.57	6.63 6.91)	—
Vb′	N-Benzoyl-DL-alanine *p*-amidinophenyl ester	8—9	28	182—184 (EtOH–ether)	$C_{24}H_{25}N_3O_6S$	59.61 (59.51	5.21 5.20	8.69 8.56	6.63 6.53)	—
Ve′	N-Benzoyl-DL-phenylalanine *p*-amidinophenyl ester	1—2	55	175—177 (EtOH–ether)	$C_{30}H_{29}N_3O_6S$	64.39 (64.22	5.22 5.19	7.51 7.43	5.73 5.81)	—
VIa	N-Benzyloxycarbonyl-β-alanine *p*-amidinophenyl ester	4	50	168—170.5 (CH_3CN)	$C_{25}H_{27}N_3O_7S$	58.47 (58.39	5.30 5.32	8.18 8.25	6.24 6.22)	—
IXa	β-Alanine *p*-amidinophenyl ester[a]	4—5	48	160.5—163 (EtOH)	$C_{24}H_{29}N_3O_8S_2 \cdot H_2O$	50.60 (50.69	5.13 5.32	7.37 7.30	11.25 11.43)	—
IXb	L-Alanine *p*-amidinophenyl ester[a]	6—7	25	210—213.5 (EtOH)	$C_{24}H_{29}N_3O_8S_2$	52.25 (52.18	5.30 5.25	7.62 7.58	11.62 11.41)	−1.30°
IXc	D-Alanine *p*-amidinophenyl ester[a]	6—7	23	210—212.5 (EtOH)	$C_{24}H_{29}N_3O_8S_2$	52.25 (52.27	5.30 5.23	7.62 7.52	11.62 11.55)	+1.40°
IXg	L-Valine *p*-amidinophenyl ester[a]	6—7	28	211.5—214 (EtOH–ether)	$C_{26}H_{33}N_3O_8S_2$	53.87 (53.61	5.74 5.50	7.25 7.08	11.06 10.54)	−4.88°
XIb	N-Acetyl-L-alanine *p*-amidinophenyl ester	12–13–14–11	32	162—164.5 (CH_3CN–ether)	$C_{19}H_{23}N_3O_6S$	54.15 (54.02	5.56 5.42	9.97 9.82	7.61 7.48)	−49.3°
XIc	N-Acetyl-D-alanine *p*-amidinophenyl ester	12–13–14–11	28	162—164 (CH_3CN–ether)	$C_{19}H_{23}N_3O_6S$	54.15 (54.02	5.50 5.42	9.97 9.90	7.16 7.71)	+51.0°
XId	N-Acetylglycine *p*-amidinophenyl ester	10—11	44	172—174 (EtOH–ether)	$C_{18}H_{21}N_3O_6S$	53.06 (52.98	5.20 5.14	10.31 10.30	— —)	—
XIbd	N-Acetyl-L-alanylglycine *p*-amidinophenyl ester	12–13–14–11	26	182—184 (EtOH–ether)	$C_{21}H_{26}N_4O_7S$	52.71 (52.78	5.48 5.51	11.71 11.51	6.70 6.66)	−32.0°
XIcd	N-Acetyl-D-alanylglycine *p*-amidinophenyl ester	12–13–14–11	20	182—184 (EtOH–ether)	$C_{21}H_{26}N_4O_7S$	52.71 (54.46	5.48 5.51	11.71 11.41	6.70 6.69)	+33.0°
XIdd	N-Acetylglycylglycine *p*-amidinophenyl ester	10–11	30	182.5—185 (EtOH–ether)	$C_{20}H_{24}N_4O_7S$	51.72 (51.89	5.21 5.09	12.06 12.01	6.90 7.00)	—
XVe	N-(1-Dimethylamino-5-naphthalenesulfonyl)-L-phenylalanine *p*-amidinophenyl ester[a]	16–17	35	159.5—161 (CH_3CN)	$C_{42}H_{44}N_4O_{10}S_3$	58.59 (58.47	5.15 5.12	6.51 6.51	11.17 11.08)	−56.8°
XVf	N-(1-Dimethylamino-5-naphthalenesulfonyl)-D-phenylalanine *p*-amidinophenyl ester[a]	16–17	28	159—161 (CH_3CN)	$C_{42}H_{44}N_4O_{10}S_3$	58.59 (58.66	5.15 5.10	6.51 6.61	11.17 10.96)	+58.9°

a) Di *p*-toluenesulfonate.
b) Step numbers are shown in Chart 1.

TABLE II. Coupling Methods and Physical Data for N-Blocked *p*-Amidinophenyl Esters

Compd. No.	Compound	Reagent[a] (Step No.)	Yield (%)	mp (°C) (from)	Formula	Analysis (%) Calcd (Found) C	H	N	$[\alpha]_D^{20}$ (c=1.0, acetone)
VIIb	N-Benzyloxycarbonyl-L-alanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	36	136—137.5 (benzene)	$C_{26}H_{25}N_3O_6$	65.68 (65.53	5.30 5.18	8.84 8.69)	−26.5°
VIIc	N-Benzyloxycarbonyl-D-alanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	35	135—137 (benzene)	$C_{26}H_{25}N_3O_6$	65.68 (65.80	5.30 5.40	8.84 8.86)	+27.0°
VIIe	N-Benzyloxycarbonyl-L-phenylalanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	49	146.5—149 (benzene)	$C_{32}H_{29}N_3O_6$	69.68 (69.71	5.30 5.16	7.62 7.58)	−8.8°
VIIf	N-Benzyloxycarbonyl-D-phenylalanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	42	144—147 (benzene)	$C_{32}H_{29}N_3O_6$	69.68 (69.78	5.30 5.07	7.62 7.43)	+9.2°
VIIg	N-Benzyloxycarbonyl-L-valine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	32	124—126 (benzene)	$C_{28}H_{29}N_3O_6$	66.79 (66.61	5.80 5.83	8.34 8.30)	−15.6°
VIIh	β-Benzyl-N-benzylouycarbonylaspartic acid N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	39	120—122 (benzene)	$C_{33}H_{31}N_3O_8$	66.32 (66.58	5.23 5.12	7.03 6.70)	−15.5°
VIIi	N-Benzyloxycarbonyl-L-proline N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	22	135—137 (benzene)	$C_{28}H_{27}N_3O_6$	67.06 (67.13	5.43 5.41	8.38 8.30)	−61.8°
VIIj	N^{α},N^{ϵ}-Bis(benzyloxycarbonyl)-L-lysine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	CP–NEt_3 (6)	30	91—93 (benzene)	$C_{37}H_{38}N_4O_8$	66.65 (66.64	5.74 5.68	8.40 8.29)	−10.5°
VIIIb′	N-Benzoyl-DL-alanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	DCC (8)	26	166.5—167.5 (benzene)	$C_{25}H_{23}N_3O_5$	67.04 (66.75	5.20 5.25	9.43 9.23)	—
VIIIe′	N-Benzoyl-DL-phenylalanine N′-*t*-butyloxycarbonyl-*p*-amidinophenyl ester	DCC (8)	83	103—105 (benzene)	$C_{28}H_{29}N_3O_5$	68.97 (68.76	6.00 5.95	8.62 8.60)	—
Xd	N-Acetylglycine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	ECC–NEt_3 (10)	72	150—152 (benzene–*n*-hexane)	$C_{19}H_{19}N_3O_5$	—	—	—	—
Xdd	N-Acetylglycylglycine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	ECC–NEt_3 (10)	35	191—193 (benzene)	$C_{21}H_{22}N_4O_6$	—	—	—	—
XIIb	N-*t*-Butyloxycarbonyl-L-alanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	ECC–NEt_3 (12)	76	158—159 (benzene)	$C_{3}H_{27}N_3O_6$	62.57 (62.26	6.16 6.25	9.52 9.39)	−37.0°
XIIc	N-*t*-Butyloxycarbonyl-D-alanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	ECC–NEt_3 (12)	65	158—159 (benzene)	$C_{23}H_{27}N_3O_6$	62.57 (62.51	6.16 6.18	9.52 9.60)	+39.5°
XIIbd	N-*t*-Butyloxycarbonyl-L-alanylglycine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	piv.Cl-py-EP (12)	35	110—111.5 (benzene–*n*-hexane)	$C_{22}H_{24}N_4O_7$	—	—	—	—
XIIcd	N-*t*-Butyloxycarbonyl-D-alanylglycine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	piv.Cl-py-EP (12)	30	110—112 (benzene–*n*-hexane)	$C_{22}H_{24}N_4O_7$	—	—	—	—
XIId	N-*t*-Butyloxycarbonylglycine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	DCC–DAP (12)	85	137—138 (benzene)	$C_{22}H_{25}N_3O_6$	61.82 (62.01	5.90 5.95	9.83 9.77)	—
XIVb	N-(1-Dimethylamino-5-naphthalenesulfonyl)-glycine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	— (12–13–14)	37[b]	166—167 (dec.) (benzene)	$C_{29}H_{28}N_4O_6S$	62.13 (62.31	5.03 4.91	9.99 9.87)	—
XIVc	N-(1-Dimethylamino-5-naphthalenesulfonyl)-L-alanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	— (12–13–14)	20[b]	132—133 (dec.) (ether)	$C_{30}H_{30}N_4O_6S$	62.70 (62.56	5.26 5.24	9.75 9.44)	−29.9°
XIVd	N-(1-Dimethylamino-5-naphthalenesulfonyl)-D-alanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	— (12–13–14)	26[b]	132—133 (dec.) (ether)	$C_{30}H_{30}N_4O_6S$	62.70 (62.89	5.26 5.24	9.75 9.68)	+30.5°
XIVe	N-(1-Dimethylamino-5-naphthalenesulfonyl)-L-phenylalanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	piv.Cl-py-EP (16)	23	177—178.5 (dec.) (acetone)	$C_{36}H_{34}N_4O_6S$	—	—	—	—
XIVf	N-(1-Dimethylamino-5-naphthalenesulfonyl)-D-phenylalanine N′-benzyloxycarbonyl-*p*-amidinophenyl ester	piv.Cl-py-Ep (16)	29	177.5—178.5 (dec.) (acetone)	$C_{36}H_{34}N_4O_6S$	—	—	—	—

a) ECC; ethyl chloroformate, NEt_3; triethylamine, CP; 1-methyl-2-chloropyridine iodide, piv. Cl,; pivaloyl chloride, EP; N-ethylpiperidine, DAP; 4-dimethylaminopyridine, py; pyridine, DCC; dicyclohexylcarbodiimide.

b) Total yield.

(100 ml), with stirring. After standing for 2 hr at room temperature, the solvent was removed. The precipitate obtained upon addition of water was recrystallized from benzene–*n*-hexane. N,O-Bis(benzyloxycarbonyl)-*p*-amidinophenol (11.7 g, 97%) was obtained as colorless needles, mp 113—115°. N-(Benzyloxycarbonyl)-*p*-amidinophenol was obtained by hydrolysis in the manner described for N,O-bis(*t*-butyloxycarbonyl)-*p*-amidinophenol. Recrystallization from ethanol gave colorless prisms, mp 188—190° (dec.), 81% yield. *Anal.* Calcd for $C_{22}H_{22}N_2O_6S$ (*p*-toluenesulfonate): C, 59.71; H, 5.01; N, 6.33. Found: C, 60.02; H, 4.99; N, 6.36.

Step 1——An ice-cold solution of benzoyl-amino acid (1 mmol) and I (236 mg, 1 mmol) in acetonitrile (20 ml) was treated with dicyclohexylcarbodiimide (DCC) (227 mg, 1.2 mmol), and the reaction mixture was allowed to stand at room temperature overnight. Dicyclohexylurea formed was separated by filtration. The filtrate was evaporated to dryness and the residue was recrystallized.

Step 2——A solution of IV (1.4 mmol) in AcOH (2 ml) was added to 25% HBr-AcOH (16 ml), and the reaction mixture was kept at room temperature overnight. The solvent was evaporated off, then the residual oil was redissolved in acetonitrile, and *p*-toluenesulfonic acid monohydrate (1.2 g, 6 mmol) was added. After

standing at room temperature for 1 hr, abs. ether was added. The resulting precipitate was subjected to recrystallization.

Step 3——Ethyl chloroformate (109 mg, 1 mmol) was added to a solution of benzoyl-amino acid (1 mmol) and triethylamine (112 mg, 1.1 mmol) in abs. tetrahydrofuran (THF) with stirring at −10°. After stirring for 15 min at −10°, a solution of II (*p*-toluenesulfonate) (246 mg, 0.8 mmol) in dry DMF was added. The reaction mixture was kept at −10° for 1 hr, then at room temperature for 2 hr. Triethylamine hydrochloride was filtered off, and the filtrate was evaporated to dryness.

Step 4——The procedure followed that of step 3, using N-benzyloxycarbonyl-amino acid in place of N-benzoyl-amino acid.

Step 5——A solution of VI (1 mmol) in methanol (60 ml) containing *p*-toluenesulfonic acid monohydrate (290 mg, 1 mmol) was hydrogenated over 10% Pd-charcoal (100 mg) for 2 hr. The catalyst was filtered off, the filtrate was evaporated to dryness, and abs. ether was added. The resulting precipitate was recrystallized.

Step 6——Coupling was carried out by a modification of the method of Mukaiyama *et al.*[5] A solution of N-benzyloxycarbonyl-amino acid (1 mmol), III (270 mg, 1 mmol) and triethylamine (242 mg, 2.4 mmol) in DMF (2 ml) was added to a suspension of 1-methyl-2-chloropyridine iodide (307 mg, 1.2 mmol) in DMF (3 ml). The reaction mixture was allowed to stand at room temperature overnight. After removal of the solvent under reduced pressure below 40°, the residue was chromatographed on a silica gel column (eluent, ethyl acetate) and recrystallized.

Step 7——Removal of the benzyloxycarbonyl groups at the amidino and amino nitrogens was carried out in the same manner as in step 5.

Step 8——This followed step 1, using III instead of I.

Step 9——Removal of the benzyloxycarbonyl group at the amidino nitrogen atom was carried out in the same manner as in step 5.

Step 10——This followed step 3, using acetyl-amino acid and III instead of benzoyl-amino acid and II.

Step 11——Compound X was used instead of VIII, following the procedure of step 9.

Step 12——A)[6] A solution of *t*-butyloxycarbonyl-amino acid (1 mmol), III (270 mg, 1 mmol), 4-dimethylaminopyridine (24 mg, 0.2 mmol) and DCC (216 mg, 1.05 mmol) in acetonitrile (3 ml) and DMF (3 ml) was kept at room temperature overnight. The resulting dicyclohexylurea was filtered off and the filtrate was evaporated to dryness. The residual oil was solidified using benzene–*n*-hexane mixture and recrystallized.

B) The next procedure followed step 4, using N-*t*-butyloxycarbonyl-amino acid and III instead of N-benzyloxycarbonyl-amino acid and II.

C)[7] A solution of N-*t*-butyloxycarbonyl-dipeptide (1 mmol), dry pyridine (79 mg, 1 mmol), N-ethylpiperidine (113 mg, 1 mmol) and 4-dimethylaminopyridine (24 mg, 0.2 mmol) in abs. THF (3 ml) was treated with pivaloyl chloride (121 mg, 1 mmol) at −10° under stirring. After stirring for a further 20 min at −10°, a solution of III (270 mg, 1 mmol) in dry pyridine (2 ml) was added. The reaction mixture was kept at −10° for 2 hr, then at room temperature overnight. After filtration, the filtrate was evaporated to dryness. The residue was solidified by trituration with acetone.

Step 13——A)[8] *p*-Toluenesulfonic acid monohydrate (2.88 g, 15.1 mmol) in abs. THF (10 ml) was added to an ice-cold solution of XII (3.03 mmol) in abs. THF (35 ml) over a period of 30 min. The solution was kept at room temperature overnight, then evaporated to dryness. The residual oil was triturated with abs. ether and solidified. The product was used for subsequent reaction without further purification.

B) Method A) was modified by replacing *p*-toluenesulfonic acid with HCl-ethyl acetate (4.3 mmol/ml, 1.2 ml).

Step 14——An ice-cold suspension of XIII (di-*p*-toluenesulfonate) (5 mmol) in N-methylmorpholine (1.52 g, 15 mmol) and abs. THF (25 ml) was treated with acetyl chloride (394 mg, 5 mmol) with stirring. The stirring was continued for 1 hr under cooling. Insoluble material was filtered off and the filtrate was evaporated to dryness. The residual oil was triturated with acetonitrie, and recrystallized.

Step 15——1-(Dimethylamino)-5-naphthalenesulfonyl chloride (0.81 g, 3 mmol) was added to an ice-cold suspension of XIII (di-*p*-toluenesulfonate) (3 mmol), N-methylmorpholine (0.95 g, 6.6 mmol) and 4-dimethylaminopyridine (0.07 g, 0.6 mmol) in abs. THF (60 ml) with stirring. The stirring was continued at 0° for 1 hr, then at room temperature overnight. The insoluble fraction was filtered off, and the filtrate was evaporated to dryness. The residual oil was separated by thin-layer chromatography, developing with ether, and recrystallized.

Step 16——The procedure followed that of step 12 C) but using N-(1-dimethylamino-5-naphthalenesulfonyl)-amino acid (1 mmol) instead of N-*t*-butyloxycarbonyl-dipeptide and omitting 4-dimethylaminopyridine.

Step 17——Compound XIV was used instead of VIII, following the procedure of step 9.

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Kinetic Measurements——Bovine trypsin was purchased from Worthington Biochemical (lot TRL). The operational molarity of the enzyme preparation was determined by the titration method of Shaw *et al.*[9] The acylation rates of trypsin by *p*-amidinophenyl esters were analyzed using a Union Giken RA/401 stopped-flow spectrophotometer and deacylation rates were determined using a Hitachi UV 200-10 double beam spectrophotometer, following the reported procedure.[3a] The reactions were monitored by measuring the liberation of *p*-amidinophenolate ions at pH 8.0 ($\Delta\varepsilon_{305nm}$: 14000).

Acknowledgement We are grateful to the staff of the Center of Instrumental Analysis of this University for elemental analysis. This work was supported in part by grants from Ministry of Education, Science and Culture, the Yamada Science Foundation, and the Foundation of Research for Medicinal Resources.

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[Chem. Pharm. Bull. 28(6)1905—1908(1980)]

Synthesis of 9-Substituted 8-Phenyltheophyllines

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(Received December 17, 1979)

Various 9-substituted 8-phenyltheophyllines (III) were prepared in two steps starting from 5,7-dimethyl-2-phenyloxazolo[5,4-*d*]pyrimidine-4,6(5H,7H)-dione (I). Thus, treatment of I with amines afforded 5-(N-substituted benzamidino)-1,3-dimethylbarbituric acids (II). The reaction of II with thionyl chloride or phosphorus oxychloride gave III.

Keywords——5,7-dimethyl-2-phenyloxazolo[5,4-*d*]pyrimidine-4,6(5H,7H)-dione; amines; 5-(N-substituted benzamidino)-1,3-dimethylbarbituric acids; thionyl chloride; phosphorus oxychloride; 9-substituted 8-phenyltheophyllines

We have recently described the synthesis of oxazolo[5,4-*d*]pyrimidines and their conversion into thiazolo[5,4-*d*]pyrimidines.[2] As part of a program directed towards the further exploitation of oxazolo[5,4-*d*]pyrimidines as synthetic intermediates, we now report the conversion of 5,7-dimethyl-2-phenyloxazolo[5,4-*d*]pyrimidine-4,6(5H,7H)-dione (I) into various 9-substituted 8-phenyltheophyllines (III) *via* 5-(N-substituted benzamidino)-1,3-dimethylbarbituric acids (II). Because of the therapeutic importance of theophylline, extensive studies have been carried out on the preparation of its derivatives; however, the synthesis of 9-substituted theophyllines has not been widely investigated.[3]

5-(N-Substituted Benzamidino)-1,3-dimethylbarbituric Acids

Refluxing of I with an excess of the appropriate arylamines in ethanol for 3 hr afforded the corresponding 5-(N-arylbenzamidino)-1,3-dimethylbarbituric acids (IIa—e) in 59—81% yields. Although the structures of IIa—e are isomeric with those of 6-arylamino-5-benzoyl-

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