CHROMOGENIC SUBSTRATES FOR PAPAIN WITH A C-TERMINAL S-BENZYLCYSTEINE RESIDUE

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Received December 15th, 1987
Accepted April 2nd, 1988

Two types of chromogenic substrates for papain were prepared, namely *p*-nitroanilides of N-acetyltripeptides of general formula Ac-Leu-Leu-A-NAn, where A = Gly, Ala, Leu, and *p*-nitroanilides of 3-carboxypropionyldi- and tripeptides of general formula Suc-B-Cys(Bzl)-NAn, where B = Gly, Gly-Gly, Ala, Ala-Ala, Leu, Leu-Leu, Gly-Leu, Leu-Ala and Ala-Met. The values of K_m, k_{cat} and C (k_{cat}/K_m) for these substrates were determined. Suc-Leu-Leu-Cys(Bzl)-NAn is the best substrate for papain its C being 60 000 $mol^{-1}\,l\,s^{-1}$.

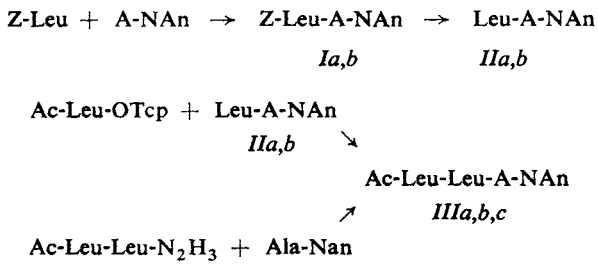
Chromogenic substrates of the *p*-nitroanilide type of amino acids and peptides[1-3] were introduced in our earlier studies describing synthetic substrates of proteolytic enzymes. We have extended this series of substrates in this study by synthesizing new chromogenic substrates for papain; the use of these substrates in food industry has been described elsewhere[4]. The design of the first group of papain substrates was based on known findings underlining the advantages of the presence of a hydrophobic amino acid, e.g. alanine in P_1 (refs[5,6]). To interpret the function of the side chain of the residue in P_1 we extended this group of substrates by synthesizing compounds with glycine or leucine in P_1 in addition to alanine*. Following this line of approach we prepared the *p*-nitroanilides of acetylleucyl-leucyl-glycine (*IIIa*), acetylleucyl--leucyl-leucine (*IIIb*) and acetylleucyl-leucyl-alanine (*IIIc*).A disadvantage observed with these substrates was that they were hydrolyzed insignificantly by papain and that they were very little soluble.

We have therefore designed another type of substrates with an N-terminal hydrophilic group, i.e. 3-carboxypropionic, the so-called succinyl residue (Suc). We have incorporated simultaneously an S-benzylcysteine residue into P_1, and in P_2 and P_3

* The symbols for amino acids and peptides comply with the suggestions of the IUPAC--IUB Commission on Biochemical Nomenclature[7,8]. All amino acids are of L-configuration (with the exception of glycine). NAn *p*-nitroanilide, Suc 3-carboxypropionyl.

resp. a glycine, alanine, leucine and methionine residue or their combinations. We prepared the *p*-nitroanilides of 3-carboxypropionylglycyl-S-benzylcysteine (*Va*), 3-carboxypropionylglycyl-glycyl-S-benzylcysteine (*Vb*), 3-carboxypropionylalanyl-S--benzylcysteine (*Vc*), 3-carboxypropionylleucyl-S-benzylcysteine (*Vd*), 3-carboxypropionylleucyl-leucyl-S-benzylcysteine (*Ve*), 3-carboxypropionylglycyl-leucyl-S-benzylcysteine (*Vf*), 3-carboxypropionylleucyl-alanyl-S-benzylcysteine (*Vg*), and carboxypropionylalanyl-methionyl-S-benzylcysteine (*Vh*). We have also assayed the *p*-nitroanilides synthesized earlier for comparison, i.e. 3-carboxypropionylalanyl--alanyl-S-benzylcysteine (*Vi*) and 3-carboxypropionylalanyl-alanyl-leucine (*Vj*) (ref.[2]).

The *p*-nitroanilides of N-acetyltripeptides were obtained by the method of stepwise synthesis (*IIIa* and *IIIb*) or by fragment condensation (*IIIc*), cf. Scheme 1.



In formulae *I*—*III*: *a*, A = Gly; *b*, A = Leu; *c*, A = Ala

SCHEME 1

The protected dipeptides were prepared by the carbodiimide method. The subsequent decarbobenzoxylation was effected by hydrogen bromide in acetic acid and the free dipeptides were set free from the corresponding hydrobromides by 10% ammonia. The synthesis of *IIIa* and *IIIb* was effected by the method of active esters (N-acetylleucine 2,4,5-trichlorophenyl ester) or by the azide method (*IIIc*) from N-acetylleucyl-leucine hydrazide, which had been obtained by hydrazinolysis of the corresponding methyl ester. The latter was obtained by the carbodiimide method using N-hydroxysuccinimide[9] from N-acetylleucine and leucine methyl ester. The key substance for the synthesis of the S-benzylcysteine derivatives was S-benzylcysteine *p*-nitroanilide[2], cf. Scheme 2.

The synthesis of dipeptides *IVa*—*d* and of tripeptide *IVh* was carried out by the carbodiimide method and the synthesis of tripeptides *IVe*—*g* by the azide method. The decarbobenzoxylation of protected di- and tripeptides *IVa*—*g* was effected by hydrogen bromide in acetic acid, the removal of the Boc group (*IVh*) by hydrogen chloride in acetic acid. The bases were liberated from the corresponding hydrobromides and from the hydrochloride by medium basic anion exchanger (Zeolite G in OH^- form) and the acylation by the carboxypropionyl residue was effected by

succinic anhydride in dimethylformamide at 80°C. The analytical data and the yields of the substrates and intermediates are given in Table I, the kinetic constants are listed in Table II.

$$\text{Z-A} + \text{Cys(Bzl)-NAn} \rightarrow \underset{IVa-d}{\text{Z-A-Cys(Bzl)-NAn}}$$

$$\text{Z-A-N}_2\text{H}_3 + \text{Cys(Bzl)-NAn} \rightarrow \underset{IVe-g}{\text{Z-A-Cys(Bzl)-NAn}}$$

$$\text{Boc-Ala-Met} + \text{Cys(Bzl)-NAn} \rightarrow \underset{IVh}{\text{Boc-A-Cys(Bzl)-NAn}}$$

$$IVa-h \rightarrow \underset{Va-h}{\text{Suc-A-Cys(Bzl)-NAn}}$$

In formulae *IV*, *V*: *a*, A = Gly; *b*, A = Gly-Gly; *c*, A = Ala; *d*, A = Leu; *e*, A = Leu-Leu; *f*, A = Gly-Leu; *g*, A = Leu-Ala; *h*, A = Ala-Met

SCHEME 2

The substrates with an N-terminal anionic residue (3-carboxypropionic) and with an S-benzylcysteine residue at the C-terminal end represent a new type of substrates for papain (and most likely also for other cysteine proteinases). Our design of the substrates for papain was based on the observation that the active site of papain can accommodate 3 to 4 binding sites of the substrates[10]. We have therefore limited our design of this series to the synthesis of N-acylated di- and tripeptides. The substrates with three amino acid residues are cleaved better than the dipeptides, the enzymatic cleavage being significantly affected by the presence of hydrophobic residues in P_2 and P_3. The order in which the substrates are cleaved is *Va* < *Vi* < *Ve*, i.e. sequence Gly-Gly < Ala-Ala < Leu-Leu. Substrate Suc-Leu-Leu-Cys(Bzl)-NAn (*Ve*) is the best substrate with an aryl residue ever synthesized for papain (C = 60 000 $mol^{-1}\,l\,s^{-1}$) and is comparable to the best substrate reported so far, i.e. Z-Phe-Cit-NAn (C = 19 100 $mol^{-1}\,l\,s^{-1}$) (ref.[11]).

The significance of the anionic residue of the papain substrates has not been discussed in literature so far. It is possible that the anionic residue may contribute by ε-amino group of lysine whose number in papain is 10 (ref.[12]). The decisive role in the optimization of the papain substrates plays the S-benzylcysteine residue in P_1 (ref.[13]). This finding is most likely of general validity not only for the hydrolysis of papain substrates but also for the enzymatic synthesis of peptides catalyzed by papain, as observed by Čeřovský and Jošt[14,15]. Interest deserves the fact that an investigation

TABLE I

N-Acylated and free *p*-nitroanilides of peptides

Compoud (Cryst. solvent[a])	M.p., °C Yield	$[\alpha]_D^{20}$ [b]	Formula (M. w.)	Calculated/Found		
				%C	%H	%N
Ia	141–142	+15·5°	$C_{22}H_{26}N_4O_6$	59·71	5·92	12·66
A	53		(442·5)	59·41	5·98	12·94
Ib	80–82	−55·3°	$C_{26}H_{34}N_4O_6$	62·63	6·87	11·24
B	59		(498·6)	62·68	7·08	11·30
IIa	204–207	+8·6°	$C_{14}H_{20}N_4O_4$	54·54	6·54	18·18
C	64		(308·3)	54·65	6·63	18·27
IIb	144–147	−41·2°	$C_{18}H_{28}N_4O_4$	59·33	7·75	15·38
D	55		(364·4)	59·14	7·88	15·51
IIIa	124–127	−7·1°[c]	$C_{22}H_{35}N_5O_6$	57·01	7·61	15·11
A	71		(463·5)	56·83	7·59	14·54
IIIb	269–271	−55·0°[c]	$C_{26}H_{41}N_5O_5$	60·10	7·95	13·48
E	37		(519·6)	59·94	8·08	13·12
IIIc	227–230	−30·0°[c]	$C_{23}H_{35}N_5O_5$	57·85	7·39	14·66
E	40		(477·5)	57·97	7·69	14·34
IVa	97–99	−6·1°	$C_{26}H_{26}N_4O_6S$	59·77	5·02	10·72
F	68		(522·5)	60·25	5·55	10·95
IVb	99–102	−4·6°[c]	$C_{28}H_{29}N_5O_7S$	58·03	5·04	12·08
B	72		(579·6)	57·85	5·00	11·68
IVc	156–157	+15·0°[c]	$C_{27}H_{28}N_4O_6S$	60·44	5·26	10·44
B	65		(536·6)	60·02	5·44	10·33
IVd	147–148	+16·4°[c]	$C_{30}H_{34}N_4O_6S$	62·27	5·92	9·68
G	69		(578·6)	62·09	6·02	9·51
IVe	158–161	−5·0°[c]	$C_{36}H_{45}N_5O_7S$	62·50	6·56	10·12
E	80		(691·8)	61·77	6·46	9·96
IVf	177–180	−31·0°[c]	$C_{32}H_{37}N_5O_7S$	60·46	5·87	11·02
E	71		(635·7)	60·22	5·91	11·20
IVg	151–153	−8·7°[c]	$C_{33}H_{39}N_5O_7S$	61·01	6·05	10·78
B	69		(649·7)	60·42	6·12	10·68
IVh	129–132	−32·0°	$C_{29}H_{39}N_5O_7S_2$	54·96	6·20	11·05
A	82		(633·8)	55·10	6·20	10·99
Va	169–172	−5·9°	$C_{22}H_{24}N_4O_7S.1/2H_2O$	53·04	5·06	11·25
D	58		(498·2)	53·26	4·93	11·68
Vb	193–194	−12·9°[c]	$C_{24}H_{27}N_5O_8S$	52·81	4·99	12·83
H	63		(545·9)	52·28	4·96	12·59

TABLE I
(Continued)

Compound (Cryst. solvent[a])	M.p., °C Yield	$[\alpha]_D^{20}$ [b]	Formula (M. w.)	Calculated/Found %C	%H	%N
Vc	180–183	−8·1°[c]	$C_{23}H_{26}N_4O_7S$	54·98	5·22	11·15
E	67		(502·5)	54·80	5·45	11·09
Vd	183–185	−47·0	$C_{26}H_{32}N_4O_7S$	57·26	5·91	10·27
F	53		(545·3)	57·65	5·84	10·27
Ve	131–133	−41·7°[c]	$C_{32}H_{43}N_5O_8S.H_2O$	56·88	6·71	10·36
D	56		(675·7)	56·84	6·70	10·21
Vf	128–131	−38·6°	$C_{28}H_{35}N_5O_8S$	55·91	5·86	11·64
D	61	22	(601·6)	56·26	6·02	11·15
Vg	225–227	22·8°	$C_{25}H_{32}N_5O_5S$	56·58	6·06	11·38
D	47		(615·7)	56·29	6·61	11·41
Vh	138–141	−32·4°[c]	$C_{28}H_{35}N_5O_8S_2$	53·07	5·57	11·05
I	51		(633·7)	53·57	5·75	10·96

[a] A Ethyl acetate–light petroleum, B 2-propanol–light petroleum, C methanol, D methanol–water, E 2-propanol, F 2-propanol–water, G 3-methyl-1-butanol, H dimethylformamide–2-propanol, I ethyl acetate; [b] in methanol; [c] in dimethylformamide.

of papain inhibition by alkyl, aryl and aralkyl isothiocyanates showed that the strongest effect has the benzyl group[16]. The significance of the S-benzylcysteine residue in positions P_2 and P_3 of the papain substrates will be considered in another study.

EXPERIMENTAL

Determination of Kinetic Constants

Stock papain solution: Papain (Serva, 2× crystallized) was dissolved in redistilled water; the concentration of the solution varied over the range $5{\cdot}1 \cdot 10^{-4} - 5{\cdot}4 \cdot 10^{-2}$ mmol l^{-1}.

The determination of the Michaelis constant K_m was carried out according to Lineweaver and Burk in the concentration range 0·25–3 mmol l^{-1} (five concentrations). The catalytic constant k_{cat} was calculated from the equation $k_{cat} = V_{max}/E$, where E stands for the enzyme concentration in the reaction mixture.

Analytical assay: The incubation medium contained 2·7 ml of 0·2M phosphate buffer, pH 6·4, with 10 mM cysteine hydrochloride and 1 mM EDTA, 0·1 ml of the stock papain solution and 0·2 ml of substrate solution in dimethylformamide. The enzyme reaction was measured conti-

nuously in terms of the liberation of *p*-nitroaniline at 410 nm in a Perkin–Elmer 552 spectrophotometer at 25°C.

Synthetic Substrates

The melting points were determined in a Kofler block and were not corrected. The samples for analysis were dried in vacuo at 70 Pa over phosphorus pentoxide at 105°C. Compounds with melting points below 120°C were dried at room temperature. The optical rotation measurements were carried out in a Perkin–Elmer polarimeter; the concentrations of the solutions varied between 0·2 and 0·3. The evaporation of the solvents from samples was carried out generally at reduced pressure. The standard procedure of treatment of the compound involves dissolving in ethyl acetate and stepwise extraction with 1M hydrochloric acid, water, 5% sodium hydrogen carbonate, water, drying over anhydrous sodium sulfate and evaporation.

N-Acetylleucyl-leucine Methyl Ester

N,N′-Dicyclohexylcarbodiimide (10·5 g) was added to a solution of acetylleucine (8·65 g; 50 mmol) and N-hydroxysuccinimide(5·75 g; 50 mmol) cooled down to −10°C. The mixture was stirred 1 h with cooling at 0°C and then treated with a solution of leucine methyl ester in dimethylformamide (25 ml) liberated from the corresponding hydrochloride (9·1 g; 50 mmol) by N-ethylpiperidine (7 ml). The solution was stirred for one additional hour with cooling (0°C) and then left aside 1 day at room temprature. N,N′-dicyclohexylurea which had separated was filtered off, the filtrate was evaporated and the dry residue treated by the standard procedure. Crystallization from 2-propanol afforded 8·0 g (53%) of a product melting at 105–106°C. The sample for analysis was crystallized in a similar manner, m.p. 106–109°C. $[\alpha]_D^{20}$ −27·9°

TABLE II

Kinetic constants of substrates cleaved by papain

Compound	K_m, mmol l^{-1}	k_{cat}, s^{-1}	C, mmol^{-1} l s^{-1}
IIIa	1·64	10·45	6·37
IIIb	—[a]	—[a]	—[a]
IIIc	0·62	0·36	0·58
Va	5·1	0·13	0·03
Vb	3·7	0·20	0·05
Vc	5·1	5·5	1·08
Vd	1·2	10·8	9·0
Ve	0·7	41·9	59·86
Vf	1·7	27·3	16·06
Vg	0·6	2·5	4·17
Vh	1·4	62·5	44·64
Vi	5·8	20·5	3·55
Vj	3·0	0·48	0·16

[a] Cannot be evaluated, not soluble.

(*c* 0·2; methanol). For $C_{15}H_{28}N_2O_4$ (300·4) calculated: 59·98% C, 9·40% H, 9·33% N; found 60·15% C, 9·69% H, 9·0% N.

N-Acetylleucyl-leucine Hydrazide

A solution of N-acetylleucyl-leucine methyl ester (7·5 g; 25 mmol) in ethanol (20 ml) and 100% hydrazine hydrate (10 ml) was allowed to stand 1 day at room temperature. The crystalline product was then filtered off and washed with methanol. The yield was 7·1 g (95%) of the hydrazide of m. p. 177—179°C. For $C_{14}H_{28}N_4O_3 \cdot 1/2\,H_2O$ (309·4) calculated: 54·36% C, 9·45% H, 18·11% N; found 54·60% C, 9·26% H, 18·30% N.

N-Acetylleucine 2,4,5-Trichlorophenyl Ester

N,N-Dicyclohexylcarbodiimide (6.3 g) was added to a solution of N-acetyleucine (5·2 g; 30 mmol) and 2,4,5-trichlorophenol (5·95 g; 30 mmol) in dimethylformamide (50 ml) cooled down to −10°C. The mixture was allowed to stand 12 h at +3°C and acetic acid (0·3 ml) was then added. The N,N′-dicyclohexylurea which had precipitated after 1-h standing at room temperature was filtered off, the filtrate was evaporated and the dry residue treated in the standard manner. Crystallization from ethyl acetate (20 ml) and ether (40 ml) afforded 4·2 g (40%) of a product melting at 123—125°C. The sample for analysis was crystallized in an analogous manner, m.p. 129—131°C, $[\alpha]_D^{20}$ −2·0° (*c* 0·2; methanol). For $C_{14}H_{16}Cl_3NO_3$ (352·7) calculated: 47·68% C, 4·57% H, 3·97% N; found 47·22% C, 4·87% H, 3·88% N.

Benzyloxycarbonylleucyl-leucine *p*-Nitroanilide (*Ib*)

A solution of benzyloxycarbonylleucine (5·3 g; 20 mmol) in methylene chloride (25 ml) cooled to −10°C and N,N′-dicyclohexylcarbondiimide (4·2 g) were added to a solution of leucine *p*-nitroanilide (5·1 g; 20 mmol). The mixture was allowed to stand 12 h at +3°C, acetic acid (0·2 ml) was then added and N,N′-dicyclohexylurea which had precipitated after 1-h standing at room temperature was filtered off. The filtrate was evaporated and the dry residue was treated in the standard manner. Crystallization from 2-propanol (60 ml) and light petroleum (800 ml) afforded 5·6 g (56%) of a product of m.p. 87—90°C. Derivatives *Ia*, *IVa-d* and *IVh* were synthesized in an analogous manner.

Leucyl-leucine *p*-Nitroanilide (*IIb*)

A 35% (w/w) solution of hydrogen bromide in acetic acid (10 ml) was added to a solution of benzyloxycarbonylleucyl-leucine *p*-nitroanilide (2·5 g; 5 mmol) in acetic acid (10 ml). The reaction mixture was allowed to stand 1·5 h and then taken to dryness. The crude hydrobromide which had separated after the addition of ether was dried 2 h over sodium hydroxide and phosphorus pentoxide, was then dissolved in a mixture of ethanol and water (1 : 1) (20 mol) and the *p*-nitroanilide base was liberated by the addition of 10% ammonia (pH 7—8). After 12 h standing at +3°C was the reaction product filtered off (950 mg) and crystallized from a mixture of 2-propanol (20 ml) and water (40 ml). The yield was 720 mg (40%) of a product of m.p. 144—147°C. The sample for analysis was crystallized from methanol and water; the m.p. was unchanged. $[\alpha]_D^{20}$ −41·2° (*c* 0·2; methanol). For $C_{18}H_{28}N_4O_4$ (364·4) calculated: 59·33% C, 7·75% H, 15·38% N; found 59·14% C, 7·88% H, 15·51% N.

Leucyl-glycine *p*-Nitroanilide (*IIa*)

This compound was prepared in analogy to *IIb* from the corresponding benzyloxycarbonyl derivative in a yield of 39%. The sample for analysis was crystallized from water; m.p. 204—207°C. $[\alpha]_D^{20}$ +8·6° (*c* 0·2; methanol). For $C_{14}H_{20}N_4O_4$ (308·3) calculated: 54·54% C, 6·54% H, 18·18% N; found: 54·65% C, 6·63% H, 18·27% N.

N-Acetylleucyl-leucyl-leucine *p*-Nitroanilide (*IIIb*)

To a solution of leucyl-leucine *p*-nitroanilide (729 g; 2 mmol) and acetylleucine 2,4,5-trichlorophenyl ester (705 mg; 2 mmol) in dimethylformamide (20 ml), which had been stirred for 4 h at room temperature, was added an additional portion of acetylleucine 2,4,5-trichlorophenyl ester (140 mg; 0·4 mmol). The reaction solution was evaporated after 2 days and the dry residue was crystallized from 2-propanol (50 ml) and light petroleum (50 ml). The yield of the product was 380 mg (37%). Compound *IIIa* was synthesized in an analogous manner.

N-Acetylleucyl-leucyl-alanine *p*-Nitroanilide (*IIIc*)

A solution of sodium nitrite (175 mg) in water (0·7 ml) was added to a solution of hydrazide N-acetylleucyl-leucine (755 mg; 2·5 mmol) in tetrahydrofuran (30 ml) and azeotropic hydrochloric acid (1 ml) cooled down to −10°C. The reaction solution was stirred 8 min with cooling to −8°C and precooled (−20°C) ethyl acetate (50 ml) was then added. After 2 min of stirring at −10°C was the organic layer separated, extracted with precooled sodium hydrocarbonate saline, dried over anhydrous sodium sulfate and added to a solution od alanine *p*-nitroanilide (530 mg; 2·5 mmol) in tetrahydrofuran (30 ml) cooled down to −5°C. The reaction mixture was allowed to stand 12 h at +3°C, was then taken to dryness and the solid residue was crystallized from methanol (35 ml) and water (80 ml). The yield was 470 mg (40%) of a product of m.p. 223—226°C. The sample for analysis was crystallized from 2-propanol and light petroleum. Compounds *IVe—g* were synthesized in an analogous manner.

Tert-butyloxycarbonylalanyl-methionine

N,N'-Dicyclohexylcarbodiimide (11 g) was added to a solution of methionine methyl ester in dimethylformamide (50 ml), which had been released from the corresponding hydrochloride (9·98 g; 50 mmol) by N-ethylpiperidine (7 ml), and tert.-butyloxycarbonylalanine (9·45 g; 50 mmol) cooled down to −10°C. The reaction solution was stirred 2 h with cooling at 0°C and then set aside for 12 h at room temperature and treated by the standard procedure. The noncrystalline residue was dissolved in 80% methanol (50 ml) and 1M sodium hydroxide was added to the solution. After 1-h stirring was the pH of the reaction solution adjusted to pH 6—7 by 20% potassium bisulfate, methanol was distilled off, the pH of the aqueous solution adjusted to pH 1 and the product extracted with ethyl acetate, dried over anhydrous sodium sulfate and evaporated. A crystalline residue (6·7 g; 42%) was obtained. The sample for analysis was crystallized from ethyl acetate and hexane, m.p. 59—62°C. $[\alpha]_D^{20}$ −27·7° (*c* 0·2; methanol). For $C_{13}H_{24}N_2O_5S$ (320·4) calculated: 48·73% C, 7·55% H, 8·74% N; found: 49·01% C, 7·54% H, 8·50% N.

3-Carboxypropionylalanyl-S-benzylcysteine *p*-Nitroanilide (*Vc*)

Hydrogen bromide (36%) in glacial acetic acid (3 ml) was added to a solution of *IVc* (1·6 g) 3 mmol). The reaction mixture was allowed to stand 1 h at room temperature and ether (50 ml; was then added. The hydrobromide which had separated was filtered off, dried 2 h in a desiccator over phosphorus pentoxide and sodium hydroxide, then dissolved in methanol (30 ml) and the

solution deionized by medium basic anion exchanger Wofatite L 150 in OH^--form in methanol. The methanolic effluent was evaporated, the residue dried by azeotropic distillation from a mixture of methanol and benzene and finally from a mixture of tetrahydrofuran and benzene. The residue was dissolved in dimethylformamide (5 ml), the solution treated with succinic anhydride (600 mg) and the reaction mixture heated at 80°C for 2 h. The reaction solution was then evaporated, the product was precipitated by the addition of water (5 ml) and filtered off. Crystallization from 2-propanol and water afforded 720 mg (48%) of a product of m.p. 175–179°C. The sample for analysis was crystallized in an analogous manner, m.p. 189–191°C. Compounds *Va–h* were prepared by an analogous procedure; the deblocking of *IVh* was effected by hydrogen chloride in acetic acid.

We thank Mrs A. Roubalová for careful technical assistance, Mrs J. Komancová for the elemental analyses and Mrs H. Bezděková of this Institute for the optical rotation measurements.

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Translated by V. Kostka.