PAPAIN-CATALYZED SYNTHESIS OF 2-NAPHTHYLAMIDES OF N-ACYLAMINO ACIDS AND DIPEPTIDES*

Václav ČEŘOVSKÝ[a], TAŤÁNA SAKS[b] and †Karel JOŠT[a]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia and*
[b] *Institute of Experimental Cardiology, Medical Academy of Sciences of U.S.S.R., 121552 Moscow, U.S.S.R.*

Received January 19th, 1987

2-Naphthylamides of several N-acylamino acids were prepared by papain-catalyzed condensation reaction in acidic medium. Under the same conditions, papain catalyzed the synthesis of peptide bond between benzyloxycarbonylglycine and phenylalanine 2-naphthylamide and between benzyloxycarbonylserine and tyrosine 2-naphthylamide. Phenylalanine 2-naphthylamide was also acylated with benzyloxycarbonyl glycine methyl ester in an alkaline medium. For comparison, papain-catalyzed condensations of benzyloxycarbonyl-S-benzylcysteine or benzyloxycarbonylalanine with aniline and its derivatives, benzylamine, phenylhydrazine, cyclohexylamine and 1-naphthylamine, were studied.

2-Naphthylamides of amino acids and peptides** find use as chromogenic or fluorogenic substrates of proteolytic enzymes in enzymology[3,4] and histochemistry[5-8]. They are synthesized from the corresponding protected amino acids and 2-naphthylamine by the method of mixed anhydrides[9-13] or by treatment with dicyclohexylcarbodiimide[12-14]. The peptide chain is then built using classical methods of peptide chemistry.

We have found that the amide bond between an N-acylamino acid and 2-naphthylamine can be built using papain as condensing agent, similarly as in the preparation of phenylhydrazides[15] or anilides of N-acylamino acids[16,17]. Only sporadical reports[10,18] exist on cleavage of 2-naphthylamides of amino acids with papin with the aim to determine its activity. The yields of 2-naphthylamides of N-acylamino acids, prepared under catalysis with papain (Table I), depend strongly on structure of the amino acid moiety (similarly as found in refs[15,16]). It is very probable that papain will also catalyze the synthesis of 2-naphthylamides of other N-acyl-L-amino acids except proline[15]. Using double amount of the enzyme and double reaction

* Part CCI in the series Amino Acids and Peptides; Part CC: Collect. Czech. Chem. Commun. *52*, 2295 (1987).

** The nomenclature and symbols of amino acids, peptides, and protecting groups obey the published recommendations[1,2]. The amino acids used in this study are of the L-configuration.

time increased the yield of benzyloxycarbonylalanine 2-naphthylamide by 10% only. Attempts to increase the yield by using higher concentration of 2-naphthylamine were unsuccessful because of its limited solubility in the given reaction medium.

In this work we also compare the effect of structure and pK_B value[19] of some other aromatic amines, benzylamine, phenylhydrazine, cyclohexylamine, and 1-naphthylamine, on the yield of the corresponding N-acylamino acid amide obtained in the papain-catalyzed reaction (Tables II and III). We used benzyloxycarbonyl-S-benzylcysteine as amino acid derivative highly specific towards the P_1 site of papain[15], or benzyloxycarbonylalanine with the same amine concentration, reaction conditions and work-up procedure. In all cases, the chemical equilibrium was shifted in the direction of synthesis by precipitation of the arising amide from the aqueous

TABLE I

Yields, physical properties and analytical data of N-acylamino acid 2-naphthylamides (X-2-naphthylamide)

X yield, %	M.p., °C $[\alpha]_D$, deg[a]	k'	Formula (mol. wt.)	Calculated/found		
				% C	% H	% N
Z-Gly	180–181[b]	1·15	$C_{20}H_{18}N_2O_3$	71·83	5·42	8·38
16	–		(334·4)	71·54	5·44	8·39
Z-Ala	191–192[d]	1·45	$C_{21}H_{20}N_2O_3$	72·39	5·79	8·04
38 (49)[c]	−33·4		(348·4)	72·51	5·79	7·90
Z-Phe	180[e]	3·14	$C_{27}H_{24}N_2O_3$	76·39	5·70	6·60
48	+43·9[f]		(424·5)	76·02	5·71	6·56
Boc-Phe	155–156	3·05	$C_{24}H_{26}N_2O_3$	73·81	6·71	7·18
39	+62·8		(390·5)	73·52	6·66	7·23
Boc-Tyr	104–105	1·26	$C_{24}H_{26}N_2O_4.0{\cdot}5\ H_2O$	69·37	6·55	6·74
30	+62·7		(415·5)	69·83	6·35	6·75
Z-Glu	217–218[g]	1·05	$C_{23}H_{22}N_2O_5$	67·97	5·46	6·89
22	−6·8[h]		(406·4)	67·67	5·43	6·77
Z-Cys(Bzl)	170–171	5·03	$C_{28}H_{26}N_2O_3S_1$	71·46	5·57	5·95
70	+22·0		(470·6)	71·06	5·60	6·06
Z-Leu	162–163[i]	2·23	$C_{24}H_{26}N_2O_3.0{\cdot}5\ H_2O$	72·15	6·81	7·01
62	−54·7[j]		(399·5)	72·52	6·83	6·72

[a] In dimethylformamide, *c* 0·3 g/100 ml; [b] 181·5°C (ref.[13]); [c] double reaction time and enzyme amount; [d] 193·5°C (ref.[13]); [e] 175°C (ref.[13]), 173–174°C (ref.[14]); [f] −4·42° (*c* 1·4, ethyl acetate), (ref.[13]); [g] 221°C (ref.[13]); [h] −0·71° (*c* 1·7, dimethylformamide), (ref.[13]); [i] 157–158°C (ref.[9]); [j] (*c* 1, chloroform), −61·6° (*c* 1·98, chloroform), (ref.[9]).

medium (pH 4·8), containing dimethylformamide in order to dissolve the reaction components. 2-Naphthylamides were synthesized in practically the same yields as anilides; several times lower yields were observed with 1-naphthylamides, probably because of greater steric requirements of 1-naphthylamine to the P'_1 site of papain.

TABLE II

Yields, physical properties, and analytical data of aromatic amides of N-benzyloxycarbonyl-S--benzylcysteine (Z-Cys(Bzl)-NH-X)

X pK_B[a]	Yield %	M.p., °C $[\alpha]_D$, deg[b]	k'	Formula (mol. wt.)	Calculated/found % C	% H	% N
C_6H_5		142—143	2·36	$C_{24}H_{24}N_2O_3S_1$	68·55	5·73	6·66
9·37	77	+14·1		(420·5)	68·11	5·70	6·59
4-CH_3-C_6H_4		163·5	3·37	$C_{25}H_{26}N_2O_3S_1$	69·09	6·03	6·45
8·92	91	+17·2		(434·6)	69·02	6·02	6·25
3-CH_3-C_6H_4		142	3·25	$C_{25}H_{26}N_2O_3S_1$	69·09	6·03	6·45
9·27	90	+16·4		(434·6)	69·20	5·95	6·48
2-CH_3-C_6H_4		151—152	2·38	$C_{25}H_{26}N_2O_3S_1$	67·68	6·14	6·32
9·56	3·5	−8·2		.0·5 H_2O (443·6)	67·40	6·07	6·27
4-Cl-C_6H_4		149—150	4·26	$C_{24}H_{23}N_2Cl_1O_3S_1$	63·35	5·09	6·16
9·85	78	+20·2		(455)	63·22	5·13	6·16
4-Br-C_6H_4		152	4·92	$C_{24}H_{23}N_2Br_1O_3S_1$	57·72	4·64	5·61
10·14	77	+16·6		(499·4)	57·83	4·68	5·67
4-CH_3CONH-		221—222	1·21	$C_{26}H_{27}N_3O_4S_1$	64·17	5·80	8·64
-C_6H_4 —	65	+20·2		.0·5 H_2O (486·6)	64·35	5·54	8·90
4-OH-C_6H_4		159—160	1·02	$C_{24}H_{24}N_2O_4S_1$	66·03	5·54	6·42
8·18	54	+9·5		(436·5)	65·44	5·51	6·41
4-CH_3OOC-C_6H_4		186	2·91	$C_{26}H_{26}N_2O_5S_1$	65·24	5·48	5·85
—	17	+29·2		(478·6)	65·37	5·41	5·85
1-naphthyl		178—179·5	3·24	$C_{28}H_{26}N_2O_3S_1$	70·12	5·68	5·84
10·08	12	0·0[c]		.0·5 H_2O (479·6)	70·36	5·54	6·05
2-naphthyl		see Table I					
9·84	70						
$C_6H_5CH_2$		141—142	2·88	$C_{25}H_{26}N_2O_3S_1$	69·09	6·03	6·45
4·67	19	−31·3		(434·6)	69·32	6·03	6·51

[a] pK_B of the starting amine (ref.[19]); [b] in dimethylformamide, c 0·3 g/100 ml; [c] optical activity of this compound was proven by the CD spectrum.

A similar result was obtained with the series of toluidides where the *ortho*-methyl group hinders the formation of the corresponding *o*-toluidide. The possible inhibition of the enzyme by *o*-toluidine was excluded by a control experiment in which papain-catalyzed synthesis of *p*-toluidide was unaffected in the presence of the same amount of *o*-toluidine. A methyl group in the *meta*- or *para*-position has a positive effect on the reaction yield, as compared with the unsubstituted aniline. Substitution with a *para*-halogen atom did not influence the yield. The somewhat unexpected lower yield in reaction with the considerably nucleophilic 4-hydroxyaniline can be explained by an increased hydrophilicity of the product (incomplete precipitation from the reaction mixture), similarly to the case of 4'-aminoacetanilide. Substituents of the IInd class in the *para*-position decrease the yield (methyl 4-aminobenzoate) or completely prevent the formation of the amide bond as in case of 4-nitroaniline ($pK_B = 13$). Of the series cyclohexylamine ($pK_B = 3{\cdot}34$), benzylamine ($pK_B = 4{\cdot}67$), phenylhydrazine ($pK_B = 8{\cdot}79$), and aniline ($pK_B = 9{\cdot}37$), the highest amide yield (90%) was obtained with phenylhydrazine (for properties see ref.[15]), lower yield was achieved with the less basic aniline, and still lower yield with benzylamine. The most basic cyclohexylamine did not react at all. These observations suggest that amide bond formation requires conjugation of the aromatic system with the amino

TABLE III

Yields, physical properties and analytical data of aromatic amides of N-benzyloxycarbonylalanine (Z-Ala-NH-X)

X pK_B[a]	Yield %	M.p., °C $[\alpha]_D$, deg[b]	k'	Formula (mol. wt.)	Calculated/found % C	% H	% N
C_6H_5		165–166[c]	0·67	$C_{17}H_{18}N_2O_3$	68·44	6·08	9·39
9·37	42	−27·0[d]		(298·3)	68·08	6·07	9·20
4-CH_3-C_6H_4		176–177	0·93	$C_{18}H_{20}N_2O_3.0{\cdot}5\ H_2O$	67·26	6·59	8·72
8·92	54	−28·8		(321·4)	67·02	6·32	8·60
4-Cl-C_6H_4		167	1·22	$C_{17}H_{17}N_2Cl_1O_3$	61·35	5·15	8·42
9·85	41	−27·3		(332·8)	61·38	5·12	8·49
1-naphthyl		198–199	0·93	$C_{21}H_{20}N_2O_3.H_2O$	68·83	6·05	7·65
10·08	3·2	—		(366·4)	69·30	5·66	7·75
2-naphthyl		see Table I					
9·84	38						

[a] pK_B of the starting amine (ref.[19]); [b] glacial acetic acid, *c* 0·3 g/100 ml; [c] 161–163°C (ref.[20]); [d] −28·6° (*c* 5, glacial acetic acid) (ref.[20]).

group of the given amine. For amines with dissociation constants K_B ranging from 10^{-9} to 10^{-10} steric effects predominate over the nucleophilicity (basicity). A similar dependence was observed[17] for amide bond formation between benzoylglycine and aniline derivatives: Since after 1 day papain afforded substantially lower yields, the reaction time was prolonged to 30 days. In the cited case, the reactivity of the individual aniline derivatives is given first of all by the basicity of the amino group and the optimum is also in the region of K_B 10^{-9} to 10^{-10}.

The attempted papain-catalyzed synthesis of the amide from benzyloxycarbonyl-S--benzylcysteine or benzyloxycarbonylphenylalanine and 7-amino-4-methylcoumarin was unsuccessful, probably because of insolubility of this amine in the reaction medium. The synthesis failed even in a medium with higher concentration of dimethylformamide or dimethyl sulfoxide in which, however, the enzyme activity is reduced.

Benzyloxycarbonylglycyl-phenylalanine 2-naphthylamide was prepared by condensation of benzyloxycarbonylglycine with an excess of phenylalanine 2-naphthylamide in the presence of papain at pH 4·8. Under these conditions, in the end period of the reaction, HPLC revealed a concurent enzymatic cleavage (about 5%) of the starting phenylalanine 2-naphthylamide (the arising dipeptide precipitated from the reaction mixture and could not be enzymatically cleaved). In a blank experiment (in the absence of benzyloxycarbonylglycine) we observed 23% cleavage of phenylalanine 2-naphthylamide after 20 h. A 20% cleavage of leucine 2-naphthylamide with papain after 1 h in a neutral medium is described[10]. Under these conditions, benzyloxycarbonylleucine 2-naphthylamide was not cleaved at all[10]. An analogous enzymatically catalyzed reaction of benzyloxycarbonylglycyl-phenylalanine with 2-naphthylamine afforded benzyloxycarbonylglycine 2-naphthylamide instead of the expected dipeptide 2-naphthylamide as the result of transpeptidase activity of papain. Another papain-catalyzed synthesis[21] of the mentioned dipeptide was performed at pH 9 and consisted in acylation of phenylalanine 2-naphthylamide with an excess of benzyloxycarbonlyglycine methyl ester. After 15 min, HPLC showed a total consumption of the nucleophile (also in this case the product precipitated). In a blank experiment (in the absence of the acylating component), only about 1% of phenylalanine 2-naphthylamide was cleaved after 1 h. Benzyloxycarbonylseryl-tyrosine 2-naphthylamide was prepared by the papain-catalyzed reaction in an acidic medium as described above. Because of higher hydrophilicity of the product, its precipitation from the reaction mixture was slower and the concurrent cleavage of the starting amino component proceeded to a greater extent. The enzymatic synthesis of the mentioned protected dipeptides has shown that 2-naphthylamide can, under certain conditions, protect directly the carboxy group of the corresponding amino component in papain--catalyzed syntheses of peptides. This synthetic method appears to be advantageous for the synthesis of further proteolytic enzyme substrates of the2-naphthylamide type.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. The reaction mixtures were evaporated on a rotatory evaporator at bath temperature 40°C. Analytical samples were dried over phosphorus pentoxide at room temperature and 150 Pa. Thin-layer chromatography (TLC) was carried out on silica gel (Silufol plates, Kavalier, Czechoslovakia) in the systems S1: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5), S2: 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5), S3: 1-butanol–acetic acid–water (4 : 1 : 1), S4: 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6). Spots were detected by the chlorination method. HPLC was performed on a Spectra Physics SP 8 700 instrument with an SP 8 400 UV-detector and SP 4 100 integrator, on a 15 × 0·4 cm column packed with Separon SIX C-18 (7 μ), flow rate 42 ml/h, detection at 222 nm. Determination of k' values was done in a mixture of methanol–0·05% aqueous trifluoroacetic acid (3 : 1). Samples for aminoacid analysis were hydrolyzed with 6 mol l^{-1} HCl at 110°C for 20 h, the analyses were carried out on an AAA 339 analyzer (Mikrotechna, Czechoslovakia). Optical rotations were measured on a Perkin–Elmer 141 MCA polarimeter. Trifluoroacetates of amino acid derivatives were prepared from the corresponding tert-butyloxycarbonyl derivatives by treatment with trifluoroacetic acid. The papain used was purchased from Enzymase, Belgium.

Preparation of Naphthylamides and Aromatic Amides of N^{α}-Acylamino Acids

The given N-benzyloxycarbonyl- or N-tert-butyloxycarbonylamino acid (1 mmol) and naphthylamine or the aromatic amine (1·5 mmol) were dissolved in dimethylformamide (3 ml for benzyloxycarbonyl-S-benzylcysteine, 2 or 3 ml for other acylamino acids) and the solution was made up to 10 ml with 0·2 mol l^{-1} acetate buffer, pH 4·8. After addition of ethylenediaminetetraacetic acid (3 mg) and cysteine hydrochloride (10 mg), the mixture was adjusted to pH 4·8. Papain (20 mg) was added and the mixture was incubated at 38°C for 24 h. The precipitate was collected on a filter, washed successively with 1 mol l^{-1} HCl (in the case of tert-butyloxycarbonyl derivatives with an HSO_4^- buffer, pH 2), water, 5% $NaHCO_3$ and water (in the case of benzyloxycarbonylglutamic acid 2-naphthylamide only with 1 mol l^{-1} HCl and water), dried and weighed. Tert-butyloxycarbonyltyrosine 2-naphthylamide was obtained from the reaction mixture by extraction with ethyl acetate and washing with the above-mentioned aqueous solutions. After drying over sodium sulfate and evaporation, the residue was triturated with light petroleum. All the thus-obtained products were homogeneous on TLC and HPLC. For determination of physical properties and analytical data (Tables I—III) the products were crystallized from ethyl acetate–light petroleum.

Benzyloxycarbonylglycyl-phenylalanine 2-Naphthylamide

A) Ethylenediaminetetraacetic acid (1 mg) and cysteine hydrochloride (5 mg) were added to a solution of benzyloxycarbonylglycine (105 mg; 0·5 mmol) and phenylalanine 2-naphthylamide trifluoroacetate (260 mg; 0·64 mmol) in a mixture of dimethylformamide (1 ml) and 0·2 mol l^{-1} acetate buffer, pH 4·8 (4 ml). After adjusting to pH 4·8, papain (20 mg) was added and the mixture was incubated at 38°C for 24 h. The product was collected on filter, washed successively with 1 mol l^{-1} HCl, water, 5% $NaHCO_3$, water and dried; yield 170 mg (71%) of the product, m.p. 165—166°C, after crystallization from ethyl acetate–light petroleum (135 mg; 56%) m.p. 166—167°C. R_F 0·81 (S1); 0·77 (S2); 0·82 (S3); 0·80 (S4); $k' = 2{\cdot}42$, $[\alpha]_D$ +14·7° (*c* 0·3, dimethylformamide). Amino acid analysis: Gly 1·00, Phe 1·09. For $C_{29}H_{27}N_3O_4$ (481·5) calculated: 72·33% C, 5·65% H, 8·73% N; found: 72·21% C, 5·66% H, 8·62% N.

B) Ethylenediaminetetraacetic acid (0·3 mg) and cysteine hydrochloride (1 mg) were added to a solution of benzyloxycarbonylglycine methyl ester (34 mg; 0·15 mmol, oil) and phenylalanine 2-naphthylamide trifluoroacetate (41 mg; 0·1 mmol) in a mixture of dimethylformamide (350 µl) and 0·5 mol l^{-1} carbonate–bicarbonate buffer, pH 9 (650 µl). After adjusting to pH 9, papain (4 mg) was added and the mixture was stirred at room temperature for 30 min. The product was isolated as described above and had the same m.p., R_F, and k' as the compound prepared under *A*). Yield 30 mg (62%).

Benzyloxycarbonylseryl-tyrosine 2-Naphthylamide

Ethylenediaminetetraacetic acid (1 mg) and cysteine hydrochloride (5 mg) were added to a solution of benzyloxycarbonylserine (93 mg; 0·39 mmol) and tyrosine 2-naphthylamide trifluoroacetate (180 mg; 0·43 mmol) in a mixture of dimethylformamide (1 ml) and 0·2 mol l^{-1} acetate buffer, pH 4·8 (4 ml). After adjusting to pH 4·8, papain (20 mg) was added and the mixture was incubated at 38°C for 24 h. The product was taken up in ethyl acetate, the organic layer was washed successively with 1 mol l^{-1} HCl, water, 5% $NaHCO_3$ and water, dried over sodium sulfate and taken down. Crystallization from ethyl acetate–light petroleum afforded 60 mg (29%), of the product, m.p. 220–221°C. R_F 0·81 (S1); 0·68 (S2); 0·82 (S3); 0·80 (S4); $k' = 0{\cdot}90$, $[\alpha]_D$ +3·9° (*c* 0·3, dimethylformamide). Amino acid analysis: Ser 1·00, Tyr 1·07. For $C_{30}H_{29}N_3O_6$ (527·6) calculated: 68·29% C, 5·54% H, 7·96% N; found: 68·20% C, 5·52% H, 7·85% N.

We are indebted to Mrs Z. Ledvinová for optical rotation measurements, to Mr J. Zbrožek for amino acid analyses and to Dr I. Frič for the CD *spectrum. Elemental analyses were performed in the Analytical Laboratory of this Institute (Dr V. Pechanec, Head).*

REFERENCES

1. *Biochemical Nomenclature and Related Documents.* International Union of Biochemistry, London 1978.
2. *Nomenclature and Symbolism for Amino Acids and Peptides. Recommendation 1983.* Eur. J. Biochem. *138*, 9 (1984).
3. Little G. H., Starnes W. L., Behal F. J.: Methods Enzymol. *45*, 495 (1976).
4. Barret A. J., Kirschke H.: Methods Enzymol. *80*, 535 (1981).
5. Gomori G.: Proc. Soc. Exp. Biol. Med. *87*, 559 (1954).
6. Burstone M. S.: *Enzyme Histochemistry and its Application in the Study of Neoplasms*, Chapter 10, p. 395. Academic Press, New York 1962.
7. Lojda Z., Gossrau R., Schiebler T. M.: *Enzymhistochemische Methoden*, Part D, p. 169. Springer, Berlin 1976.
8. Pearse A. G. E.: *Histochemistry, Theoretical and Applied.* Vol. 2, Chapter 22, p. 962.Churchill Livingstone, Edinburgh and London 1972.
9. Green M. N., Tsou K. C., Bressler R., Seligman A. M.: Arch. Biochem. Biophys. *57*,458 (1955).
10. Folk J. E., Burstone M. S.: Proc. Soc. Exp. Biol. Med. *89*, 473 (1955).
11. Plapinger R. E., Nachlas M. M., Seligman M. L., Seligman A. M.: J. Org. Chem. *30*, 1781 (1965).
12. Glenner G. G., Cohen L. A., Folk J. E.: J. Histochem. Cytochem. *13*, 57 (1965).
13. Goldstein T. P., Plapinger R. E., Nachlas M. M., Seligman A. M.: J. Med. Pharm. Chem. *5*, 852 (1962).
14. Nesvadba H.: Monatsh. Chem. *93*, 386 (1962).

15. Čeřovský V., Jošt K.: Collect. Czech. Chem. Commun. *49*, 2557 (1984).
16. Fox S. W., Pettinga C. W., Halverson J. S., Wax H.: Arch. Biochem. Biophys. *25*, 21 (1950).
17. Waldschmidt-Leitz E., Kühn K.: Hoppe-Seyler's Z. Phsiol. Chem. *285*, 23 (1950).
18. Barrett A. J.: Anal. Biochem. *47*, 280 (1972).
19. *Handbook of Chemistry and Physics*, 62nd Edition (R. C. Weast and M. J. Astle, Eds), Part D, p. 139. CRC Press, Boca Raton, Florida 1981—1982.
20. Doherty D. G., Popenoe E. A.: J. Biol. Chem. *184*, 449 (1951).
21. Mitin Y. V., Zapevalova N. P., Gorbunova E. Y.: Int. J. Pept. Protein Res, *23*, 528 (1984).

Translated by M. Tichý.