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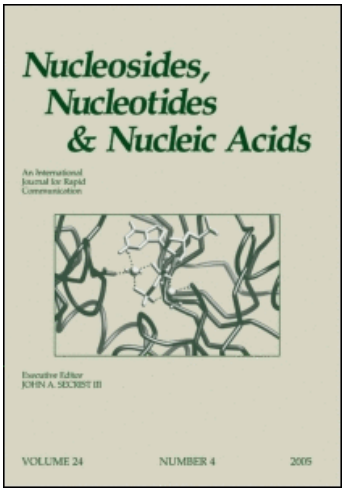

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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Oligonucleotides and Nucleotide-Peptides. LIV. The Synthesis and Hydrolytic Properties of Adenylyl-(5'→N$_\varepsilon$)-lysylpeptides — Model Compounds of the AMP-RNA Ligase Covalent Complex

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To cite this Article Juodka, Benediktas , Sasnauskiene, Sofija and Petniunaite, Ruta(1992) 'Oligonucleotides and Nucleotide-Peptides. LIV. The Synthesis and Hydrolytic Properties of Adenylyl-(5'→N$_\varepsilon$)-lysylpeptides — Model Compounds of the AMP-RNA Ligase Covalent Complex', Nucleosides, Nucleotides and Nucleic Acids, 11: 8, 1445 — 1453
To link to this Article: DOI: 10.1080/07328319208021186
URL: http://dx.doi.org/10.1080/07328319208021186

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NUCLEOSIDES & NUCLEOTIDES, 11(8), 1445-1453 (1992)

OLIGONUCLEOTIDES AND NUCLEOTIDE-PEPTIDES. LIV. THE SYNTHESIS AND HYDROLYTIC PROPERTIES OF ADENYLYL-(5'→N_ε)-LYSYLPEPTIDES -- MODEL COMPOUNDS OF THE AMP-RNA LIGASE COVALENT COMPLEX[1]

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Abstract: RNA ligase active center model compounds - adenylyl-(5→N_ε)-lysylpeptides were synthesized. The stability of these compounds in aqueous solutions was studied and it was shown, that the carboxyl group of lysine and glutamic acid or hydroxyl group of threonine have no effect upon the hydrolytic mechanism of the adenylyl-(5'→N_ε)-lysylpeptides. This led to conclusion, that the hydrolysis of the AMP-RNA ligase complex is dependant upon other amino acid functional groups, which may be located next to the phosphoamide center, as a result of tertiary protein structure.

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RNA ligase from T4 phage infected *Escherichia coli* (polinucleotidyl synthetase, EC 6.5.1.3) was first described in 1972.[2] The enzyme has been found to catalyze the ATP-dependant circularization and intermolecular joining of oligonucleotides.[3,4] This catalysis is found to occur in three stages:

1. $E + ATP \xrightleftharpoons{Mg^{2+}} E\text{-}pA + PP_i$
2. $E\text{-}pA + pN(pN)_n \rightleftharpoons [A(5')ppN(pN)_n]\cdot E$
3. $N(pN)_mpN + [A(5')ppN(pN)_n]\cdot E \rightleftharpoons N(pN)_mpNpN(pN)_n + AMP + E$

In the first stage of the reaction the RNA ligase reacts with ATP to form the covalent intermediate — AMP-RNA ligase complex (E-pA) with the liberation of free pyrophosphate.[5] Further data provided evidence for the formation of the phosphoamide bond between AMP and

[1]Abbreviations used for amino acids and peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature J. Biol. Chem. 1972 247, 977. The following additional abbreviations are used: Boc, tret-Butoxycarbonyl; Cbz, Benzyloxycarbonyl; DMF, N,N'-dimethylformamide; DMSO, dimethylsulphoxide; TEA, triethylamine; DCC, N,N'-dicyclohexylcarbodiimide.

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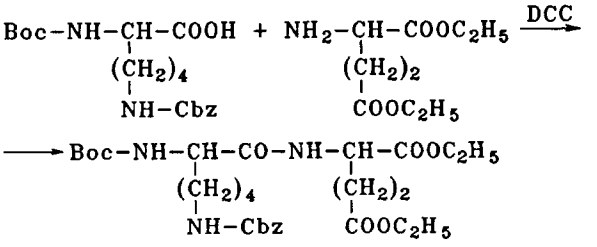
RNA ligase via lysine ε-amino group.[6] The covalent complex (E-pA) has also been found to undergo hydrolysis in aqueous solution, where adenosine and AMP are identified as decomposition products.[7]

Studies conducted with model phosphoamide type nucleotidyl-(P→N)-amino acids or nucleotidyl-(P→N)-peptides showed that phosphoester bond cleavage (adenosine elimination) occurs only under the conditions where the amino acid residue hydroxyl or carboxyl group is located next to the phosphoamide center.[8] Based upon the amino acid sequence of RNR ligase,[9,10] it has been shown that lysine-99 (to which AMP is bound) is found between threonine-98 and glutamic acid-100. The phosphoamide center of the AMP-RNA ligase covalent complex is localized between two potential functional groups which may participate in intramolecular nucleophilic catalysis. It is therefore possible that their existence is responsible for the unexpected cleavage which the phosphoamide center undergoes during the hydrolysis of the AMP-RNA ligase complex.

The purpose of this work has been to synthesize adenylyl-(5′→N_ε)-lysine and adenylyl-(5′→N_ε)-lysylpeptides, which would serve as model compounds for the AMP-RNA ligase covalent complex. Furthermore, the influence of the threonine hydroxyl and lysine or glutamic acid carboxyl groups upon the stability of the phosphoamide center was studied.

RESULTS AND DISCUSSION

The protected dipeptides Boc-Lys(Cbz)-Glu(OEt)-OEt (I) and Boc-Thr-Lys(Cbz)-OMe (II) were synthesized according to the N,N′-dicyclohexylcarbodiimide (DCC) method in solution:



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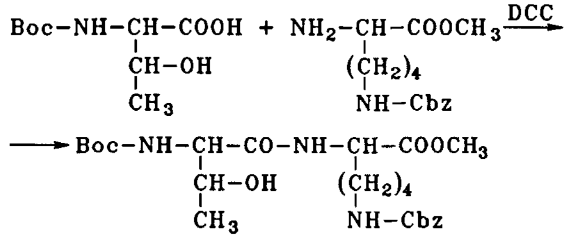
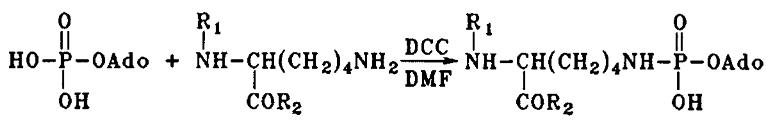
I. Boc-Lys(Cbz)-Glu(OEt)-OEt



II. Boc-Thr-Lys(Cbz)-OMe

The structure of the dipeptides (I, II) was determined by ^{1}H NMR. The compounds underwent complete acid hydrolysis (6N HCl, 100°C, 24 h), from which the amino acid ratio was determined. The lysine ε-amino groups were then deprotected by hydrogenolysis of the protected dipeptides (I,II) (catalyst — palladium over charcoal).

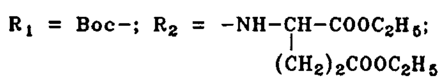
The incubation 10-molar excesses of N_α-Cbz-lysine ethyl ester or N_ε-amino group deblocked lysyldipeptide esters with AMP, in the presence of DCC, within the reaction mixture resulted in the synthesis adenylyl-(5'→N_ε)-lysine ethyl ester (III) or adenylyl-(5'→N_ε)-lysyldipeptide esters (IV, V):



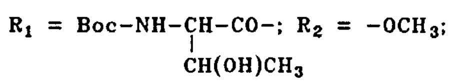
III. Adenylyl-(5'→N_ε)-(N_αCbz)-L-lysine ethyl ester

R_1 = -Cbz; R_2 = -OC_2H_5;

IV. Adenylyl-(5'→N_ε)-(N_αBoc)-L-lysyl-L-glutamic acid diethyl ester



V. Adenylyl-(5'→N_ε)-(Boc)-L-threonyl-L-lysine methyl ester



The treatment of compounds III-V with 1N sodium hydroxide (37°C, 1 h) gave the free carboxylate analogues VI-VIII:

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TABLE 1: Some characteristics of synthesized adenylyl-(5′→N_ε)-lysylpepetides (III-VIII).

Compounds	Yield,[a] %	R_f[b]		UpA[c]	Ratio adenine: phosphate: amino acids
		A	B		
Cbz-Lys(N_εpA)-OEt(III)	80	0.61	0.75	0.43	1:0.95:0.80
Boc-Lys(N_εpA)-Glu(OEt)-OEt(IV)	60	0.78	0.87	0.31	1:0.78:0.83:0.85
Boc-Thr-Lys(N_εpA)-OMe(V)	45	0.68	0.85	0.34	1:0.79:0.71:0.80
Cbz-Lys(N_εpA)-OH(VI)	100	0.22	0.31	0.76	
Boc-Lys(N_εpA)-Glu-OH(VII)	100	0.16	0.24	0.91	
Boc-Thr-Lys(N_εpA)-OH(VIII)	100	0.20	0.31	0.74	

[a]Yield of compounds III-VIII was estimated spectrophotometrically after elution from chromatographic sheets. [b]R_f in solvent systems:A - 2-propanol:conc. NH_4OH:water(7:1:2); B - ethanol:1M ammonium acetate(7:3). [c]Electrophoretic mobility relative to adenosine-5′-monophosphate (0.05M triethylamine bicarbonate buffer pH 7.5).

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VI. Adenylyl-(5′→N_ε)-(N_αCbz)-L-lysine

R_1 = -Cbz; R_2 = -OH;

VII. Adenylyl-(5′→N_ε)-(N_αBoc)-L-lysyl-L-glutamic acid

R_1 = Boc–; R_2 = –NH–CH(–$(CH_2)_2COOH$)–COOH;

VIII. Adenylyl-(5′→N_ε)-(Boc)-L-threonyl-L-lysine

R_1 = Boc–NH–CH(–$CH(OH)CH_3$)–CO–; R_2 = –OH;

Compounds III-VIII were separated from the reaction mixture by the method of paper chromatography, utilizing solvent system A (2-propanol:conc.NH_4OH:water in a volume ratio of 7:1:2). The structure of adenylyl-(5′→N_ε)-lysine ethyl ester (III) and adenylyl-(5′→N_ε)-lysylpeptide esters (IV, V) was determined by establishing the ratio of adenine, phosphate and amino acids after complete acid hydrolysis (6N HCl, 100°, 24 h) of these compounds. Structure of these compounds was also confirmed by ^1H NMR and by UV spectroscopic method, λ_{max}=259 nm (pH 7.0). These data along with the chromatographic and electrophoretic characteristics of synthesized compounds are presented in TABLE 1.

The hydrolytic stability of compounds III-VIII was studied. The percentage concentration of adenosine 5′-monophosphate formed as a function of acid concentration (0.01 - 0.2N HCl, 37°C, 1 h) is presented in FIGURE 1.

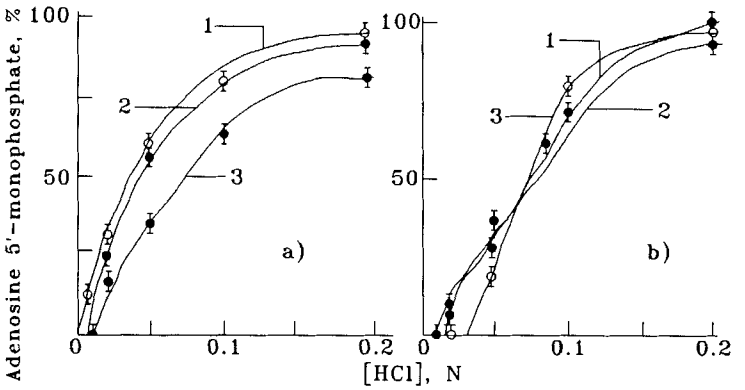

FIGURE 1. The dependance of the hydrolysis of the phosphoamide bond of compounds III-VIII upon the concentration of HCl (37°C, 1 h).

a) 1. Cbz-Lys(N_εpA)-OEt (III)
2. Boc-Lys(N_εpA)-Glu(OEt)-(OEt) (IV)
3. Boc-Thr-Lys(N_εpA)-OMe (V)

b) 1. Cbz-Lys(N_εpA)-OH (VI)
2. Boc-Lys(N_εpA)-Glu-OH (VII)
3. Boc-Thr-Lys(N_εpA)-OH (VIII)

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Studies upon the hydrolytic characteristics of adenylyl-(5'→N_ε)-lysine ethyl ester (III) and adenylyl-(5'→N_ε)-lysylpeptide esters (IV, V) determined that all they undergo hydrolysis to give AMP and the respective lysine or dipeptide esters (FIGURE 1,a). Furthermore, it is of interest to note, that their respective analogues with free carboxylate groups (VI-VIII) underwent phosphoamide bound hydrolysis under these conditions to give AMP (FIGURE 1,b). These results would indicate that the lysine α-carboxylic group (VI, VIII), the threonine hydroxylic group (V) and carboxylic groups of glutamic acid (VII) are distanced from the phosphoamide center, and for this reason do not participate in the mechanism of adenylyl-(5'→N_ε)-lysine (VI) and adenylyl-(5'→N_ε)-lysyldipeptides (VII, VIII) hydrolysis. It is speculated that these neighbouring functional groups are located in a manner which is sterically unfavorable with respect to the phosphoramide center and can not take place in the intramolecular catalysis. In summary, the results of these experiments would then indicate that the cleavage of AMP-RNA ligase complex to form adenosine does not depend upon neighbouring threonine and glutamic acid functional groups, but rather upon other such amino acid residues, which may approach the

phosphoamide center more closely, and which are due to the tertiary structure of the RNR ligase polypeptide.

These conclusions are in agreement with those of Heaphy *et al.*,[11] which found that replacement of glutamic acid-100 (Glu) with threonine (Thr) or glutamine (Gln) did not influence the formation of the AMP-RNA ligase complex on the first stage of the reaction.

EXPERIMENTAL

Materials. The following reactants were used in this work: adenosine-5'-phosphate (Na_2), L-glutamic acid, Boc-L-threonine, α-Cbz-L-lysine, α-Boc-ε-Cbz-L-lysine DCCA salt (Reanal, Hungary), DCC (Ferak, Berlin), TEA (Merck, West Germany), Dowex 50 (Serva, West Germany). For analytical purposes used plates "Silufol" UV 254 (Czechoslovakia) and Kieselgel 60 F_{254} (Merck, West Germany). The reaction mixtures were chromatographed on paper FN 12, electrophoresis was carried out on paper FN 16 (Filtrak, GDR). In general, reactions were carried out with freshly distilled and carefully dried solvents avoiding moisture completely.

Ultraviolet spectra were recorded on a spectrophotometer DU-50 (Beckman, USA). Nuclear magnetic resonance spectra were recorded on a Brucker 360 MHz instrument (West Germany) and chemical shifts are reported in parts per million downfield from tetramethylsilane.

The synthesis of glutamic acid diethyl ester and N_α-Cbz-lysine ethyl ester was carried out according to [12].

Dipeptide Synthesis

Boc-Lys(Cbz)-Glu(OEt)-OEt(I): Boc-Lys(Cbz)-OH (1.9 g, 5 mmol), Glu(OEt)-OEt•HCl (1.2 g, 5 mmol) were dissolved in methylene chloride (100 ml). Triethylamine (0.7 ml, 5 mmol) and N,N'-dicyclohexylcarbodiimide (DCC) (1.2 g, 6 mmol) were added to the solution which had been cooled in an ice-salt bath (-10°C). The mixture was then stirred at 0-4°C overnight. The dicyclohexylurea was removed by filtration and the solvent extracted with 10% aqueous solution of sodium bisulphate (3 x 50 ml), water (3 x 50 ml) and saturated aqueous sodium bicarbonate (3 x 50 ml). The organic layer was then continuously washed with distilled water until

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neutrality was obtained. The methylene chloride portion was then dried over anhydrous magnesium sulphate, after which the drying agent was removed and the solvent evaporated under reduced pressure. The resulting residue was treated with a mixture of diethyl ether and hexane, from which the precipitated product was collected, with a yield of 2.9 g (79%). ^{1}H NMR (DMSO) δ ppm: 8.15 (d, 1H, NH); 1.82 (m, 2H, $CH_{2\beta}$); 2.95 (q, 2H, $CH_{2\varepsilon}$); 1.15 (d, 3H, CH_3); 7.20 (m, 1H, NH_ε); 6.75 (d, 1H, NH_α); 2.38 (t, 2H, $CH_{2\gamma}$); 1.37 (s, 9H, Boc); 4.25 (m, 1H, CH_α); 7.35 (m, 5H, arom.).

Boc-Thr-Lys(Cbz)-OMe (II): This peptide was synthesized by the method described above. Boc-Thr (0.654 g, 3 mmol), Lys(Cbz)-OMe•HCl (0.99 g, 3 mmol) were condensed with DCC (0.7 g, 3.5 mmol) to yield 1.1 g of the dipeptide (74%). ^{1}H NMR (DMSO) δ ppm: 8.74 (d, 1H, NH); 6.16 (d, 1H, CH_α); 5.01 (d, 1H, OH); 4.19 (m, 1H, CH_β); 1.12 (d, 1H, CH_3); 1.37 (s, 9H, Boc); 7.20 (m, 1H, NH_ε); 6.75 (d, 1H, NH_α); 7.35 (m, 5H, arom.); 2.95 (q, 2H, $CH_2\varepsilon$).

Cbz-deprotection: The protected dipeptides (I, II) (2 mmol) were dissolved in ethanol (50 ml), after which palladium over charcoal (0.1 g) added and the suspension was stirred under H_2 bubbling for 2 h at room temperature. The catalyst was filtered off and the solvent was evaporated. The lysine dipeptides with free ε-amino group were then precipitated by addition of diethyl ether-hexane mixture.

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Adenylyl-(5'→N)-lysyldipeptide synthesis

Cbz-Lys(N_εpA)-OEt (III): AMP (trioctylamine salt, 0.1 mmol) was dissolved in a 2 ml solution of DMF-DMSO (anhydrous, 1:1 volume ratio), to which was added N_αCbz-Lys-OEt (0.3 g, 1 mmol), TEA (0.14 ml, 1 mmol), DCC (0.103 g, 0.5 mmol). The reaction mixture was incubated for 4 h at 60°C. Compound III was separated from the reaction mixture by the method of paper chromatography, utilizing solvent system A (2-propanol: conc. NH_4OH: water in a volume ratio of 7:1:2). Final yield was determined to be 80%. ^{1}H NMR (DMSO) δ ppm: 8.39 (s, 1H, H-8); 8.17 (s, 1H, H-2); 5.92 (d, 1H, H-1'); 3.90-4.65 (m, 5H, H-2', H-3', H-4', H-5'); 2.95 (q, 2H, $CH_{2\varepsilon}$); 1.19 (d, 3H, CH_3); 7.20 (m, 1H, NH_ε); 6.75 (d, 1H, NH_α); 7.40 (m, 5H, arom.).

Boc-Lys(N_εpA)-Glu(OEt)-OEt (IV): Compound IV was synthesized analogously to compound III. AMP (trioctylamine salt, 0.1 mmol) was condensed with the deprotected dipeptide

- Boc-Lys-Glu(OEt)-OEt (0.56 g, 1 mmol) by reaction with DCC (0.103 g, 0.5 mmol). Compound IV was also separated from the reaction mixture by paper chromatography utilizing solvent system A, with final yield 60%. ^{1}H NMR (DMSO) δ ppm: 8.38 (s, 1H, H-8); 8.17 (s, 1H, H-2); 5.92 (d, 1H, H-1'); 3.90-4.60 (m, 5H, H-2', H-3', H-4', H-5'); 8.15 (d, 1H, NH); 1.82 (m, 2H, CH_β); 2.05 (m, 1H, CH'_β); 1.15 (t, 3H, CH_3); 7.20 (m, 1H, NH_ε); 6.75 (d, 1H, NH_α); 2.95 (q, 2H, $CH_{2\varepsilon}$); 2.38 (t, 2H, $CH_{2\gamma}$); 1.37 (s, 9H, Boc).

Boc-Thr-Lys(N_εpA)-OMe (V): Compound V was synthesized like compound III. AMP (trioctylamine salt, 0.1 mmol) was condensed with deprotected dipeptide - Boc-Thr-Lys-OMe (0.378 g, 1 mmol) by reaction with DCC (0.103 g, 0.5 mmol). Compound V was also separated from the reaction mixture by paper chromatography utilizing solvent system A, with the final yield of 45%. ^{1}H NMR (DMSO) δ ppm: 8.40 (s, 1H, H-8); 8.17 (s, 1H, H-2); 5.96 (d, 1H, H-1'), 3.98-4.71 (m, 5H, H-2', H-3', H-4', H-5'); 8.75 (d, 1H, NH); 6.16 (d, 1H, CH_α); 5.01 (d, 1H, OH); 4.19 (m, 1H, CH_β); 1.12 (d, 3H, CH_3); 1.37 (s, 9H, Boc); 7.20 (m, 1H, NH_ε); 6.75 (d, 1H, NH_α); 7.35 (m, 5H, arom.); 2.95 (q, 2H, $CH_2\varepsilon$).

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Synthesis of the free carboxylate analogues (VI-VIII)

Cbz-Lys(N_εpA)-OH (VI): 1 ml aqueous solution of compound V (0.02 mmol) was added to a 1 ml solution of 2N NaOH and incubated at 37°C for 1 h. The reaction mixture was then neutralized with Dowex-50 (H^+) and compound (VI) was isolated by paper chromatography utilizing solvent system A.

Boc-Lys(N_εpA)-Glu-OH (VII) and Boc-Thr-Lys(NεpA)-OH (VIII) were prepared analogously to compound VI.

Adenylyl-(5'→N_ε)-lysylpeptide esters (III-V) and analogues with free carboxylate (VI-VIII) hydrolysis was carried out as previously described.[13]

Acknowledgments

The authors are grateful to Dr. F. Mutulis and Dr. P. Zarins for reagents and for help in peptide synthesis. We also wish to give our thanks to Dr. J. Saulytis for ^{1}H NMR spectra data (Institute of Organic Synthesis Academy of Sciences, Latvia).

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Received 5/20/90
Accepted 6/17/92

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