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PEPTIDYL TRANSFER RNA

X. THE CHEMICAL SYNTHESIS OF PEPTIDYL TRANSFER RNA CONTAINING HYDROXY AMINO ACIDS*

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SUMMARY

The synthesis of L-serine and L-tyrosine containing oligopeptidyl-tRNA is described. The method involves a reaction between *N*-hydroxysuccinimide ester of *N*-*o*-nitrophenylsulfenyl-L-serine$_n$ ($n = 1$–2) or *N*-*o*-nitrophenylsulfenyl-(L-Tyr)$_n$-Gly ($n = 1$–3) and aminoacyl-tRNA and subsequent removal of the N-protecting group by treatment with $Na_2S_2O_3$. In the case of N-protected carboxyl-activated L-Ser–L-Ser, the acylation reaction was repeated three times resulting in the formation of (L-Ser)$_6$–Phe–tRNA.

INTRODUCTION

Peptidyl-tRNA's are known to be intermediates in protein biosynthesis. It seems to us that the study of their properties as a function of the peptide chain length and amino acid composition might lead to a better understanding of the complex mechanism of protein biosynthesis. In previous publications from our laboratory, we described a method for the chemical synthesis of glycine and alanine-containing oligopeptidyl-tRNA[1–4]. The synthesis is based on the reaction between the *N*-hydroxysuccinimide ester of *N*-monomethoxytrityl amino acid or peptide and amino acyl-tRNA. The N-protecting group is removed after the acylation reaction by mild acidic treatment (5 % dichloroacetic acid at 4° for 5 min). Recently we reported on the use of *o*-nitrophenylsulfenyl (NPS) group as an N-blocking group in the synthesis of peptidyl-tRNA[5]. In this case the N-blocking group was removed after the acylation reaction by treatment with $Na_2S_2O_3$.

In the present communication the synthesis of oligopeptidyl-tRNA's containing L-serine, L-tyrosine and L-tryptophan is described.

MATERIALS AND METHODS

The tRNA (*Escherichia coli* B) used was obtained from Calbiochem, Calif., U.S.A. L-[^{14}C]amino acids were purchased from the Radiochemical Centre, Amersham, Great Britain. *N*-Hydroxysuccinimide was purchased from Aldrich, Wisc.,

Abbreviation: NPS, *o*-nitrophenylsulfenyl.

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U.S.A., and was recrystallized from ethyl acetate. NPS-amino acids were prepared according to ZERVAS *et al.*[6] and were stored in the form of their dicyclohexylammonium salts. Peptides were prepared according to the literature[6,7]. *N*-Monomethoxytrityl-L-serine and its *N*-hydroxysuccinimide ester were prepared according to the general procedure reported elsewhere[3].

All amino acids used in this work were L-amino acids.

Paper electrophoresis was performed in a high-voltage apparatus (4500 V, 45 V/cm) in which the paper was immersed in a water-cooled high-boiling petroleum fraction (versol). Two different buffers were used, one at pH 2.5 (1 M acetic acid) and the second at pH 1.9, composed of acetic acid–formic acid–water (90:30:480, by vol.). The relative electrophoretic mobilities of the different compounds are given in Table I. Amino acids and peptides were detected by ninhydrin. When radioactive compounds were chromatographed, the paper was cut into strips and the radioactivity was detected by a Packard radiochromatogram scanner Model 7201.

TABLE I

PAPER-ELECTROPHORETIC MOBILITIES OF DIFFERENT COMPOUNDS

Compound	*pH 2.5*	*pH 1.9*
Phe	1.00	1.00
Ser–Phe	1.9	1.15
Ser_2–Phe	1.45	0.90
Ser_4–Phe	—	0.80
Ser_6–Phe	—	0.70
Tyr–Phe	1.35	0.86
Tyr–Gly–Phe	1.60	0.84
Tyr_2–Gly–Phe	1.30	0.68
Tyr_3–Gly–Phe	—	0.67
Trp–Phe	—	0.86
Trp–Gly–Phe	1.50	0.76

N-Hydroxysuccinimide ester of NPS-L-serine

NPS-L-serine (17.5 g, 68 mmoles) was dissolved in dry tetrahydrofuran (200 ml) together with *N*-methylmorpholine (7.5 ml, 68 mmoles) and chilled to −13°. Isobutyl chloroformate (8.8 ml, 68 mmoles) was added, and after 30 sec, *N*-hydroxysuccinimide (8.5 g, 74 mmoles) dissolved in dry tetrahydrofuran (100 ml) was added with rapid stirring. The reaction mixture was kept at room temperature for 2 h. The precipitate was removed by filtration and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (250 ml) and successively washed with 0.1 M H_2SO_4 (100 ml), water (100 ml), 5 % $NaHCO_3$ (100 ml) and water (100 ml). Finally the aqueous layer was dried over $MgSO_4$ and then evaporated to dryness under reduced pressure. The residue was triturated with diethyl ether and the crystals were collected by filtration. For purification the material was dissolved in chloroform containing 20 % methanol (300 ml) and precipitated by adding light petroleum (b.p. 40–60°), giving 14 g (57 % yield) *N*-hydroxysuccinimide ester of NPS-L-serine, m.p. 158°.

Analysis: Calculated for $C_{13}H_{13}N_3SO_7$: C, 43.94; H, 3.66; N, 11.83; S, 9.01 %. Found: C, 44.04; H, 3.85; N, 11.4; S, 9.37 %.

N-Hydroxysuccinimide ester of NPS-L-Ser-L-Ser

A solution of L-serine (3.15 g, 30 mmoles) and $NaHCO_3$ (2.32 g, 30 mmoles) dissolved in water (300 ml) was added to a solution of *N*-hydroxysuccinimide ester of NPS-L-serine (7.2 g, 20 mmoles) in dimethoxyethane (200 ml). Water (about 5 ml) was added to clarify the solution. The reaction mixture was kept at room temperature for 2 h and the organic solvent was distilled off under reduced pressure. The residual aqueous solution was washed with ethyl acetate (100 ml), acidified to pH 3.0 by adding 1 M H_2SO_4 and extracted with ethyl acetate (4 times 150 ml). The ethyl acetate was washed with a saturated solution of NaCl (3 times 30 ml), dried over $MgSO_4$ and distilled off under reduced pressure. When the solution became concentrated, crystallization began. The crystals were collected by filtration, giving 2.5 g (yield 20 %), m.p. 155°.

A solution of NPS-L-Ser-L-Ser (4 g, 11.5 mmoles) dissolved in dry tetrahydrofuran (200 ml) together with *N*-methylmorpholin (1.25 ml, 11.5 mmoles) was chilled to −15°. Isobutyl chloroformate (1.5 ml, 11.5 mmoles) was added and immediately afterwards a solution of *N*-hydroxysuccinimide (1.5 g, 13 mmoles) in dry tetrahydrofuran (50 ml) was added. The reaction mixture was warmed to 0° for 5 min, then to 40° for 2 min and finally to room temperature. The precipitate was removed by filtration and the treatment continued as described in the synthesis of the *N*-hydroxysuccinimide ester of NPS-L-serine. The final product had a melting point of 122–123° (yield 39 %).

Analysis: Calculated for $C_{16}H_{18}N_4SO_9$: C, 43.44; H, 4.07; N, 12.67; S, 7.24 %. Found: C, 43.24; H, 4.41; N, 12.67; S, 7.8 %.

N-Hydroxysuccinimide ester of NPS-L-tyrosine

0.1 M H_2SO_4 (400 ml) was added to a suspension of NPS-L-tyrosine dicyclohexylammonium salt (85 g, 74 mmoles) in diethyl ether (200 ml). The mixture was stirred until all the material was dissolved and after 15 more min the diethyl ethereal layer was separated, washed with water, dried over $MgSO_4$ and evaporated to dryness. To the residue dissolved in ethyl acetate (300 ml), *N*-hydroxysuccinimide (10 g, 87 mmoles) and dicyclohexylcarbodiimide (15.3 g, 74 mmoles) were added. After 4 h at room temperature the dicyclohexylurea, which precipitated out, was removed by filtration. The filtrate was washed with 5 % $NaHCO_3$ (100 ml), then with water (100 ml). The solution, after drying over $MgSO_4$, was evaporated to dryness under reduced pressure. The residue was triturated with diethyl ether and the solid material was isolated by filtration. Recrystallization was done from isopropanol, giving 21 g (66 % yield) *N*-hydroxysuccinimide ester of NPS-L-tyrosine, m.p. 152–153°.

Analysis: Calculated for $C_{19}H_{17}N_3SO_7$: C, 52.90; H, 3.95; N, 9.74; S, 7.42 %. Found: C, 52.8; H, 4.12; N, 9.23; S, 7.14 %.

N-Hydroxysuccinimide ester of NPS-Tyr-Gly

A solution of *N*-hydroxysuccinimide ester of NPS-L-tyrosine (4 g, 9 mmoles) in dimethoxyethan (200 ml) was added to a solution of glycine (1.35 g, 18 mmoles) and $NaHCO_3$ (1.52 g. 18 mmoles) in water (80 ml). The reaction mixture was kept at room temperature for 2 h. The organic solvent was removed by distillation under reduced pressure and the aqueous residue was washed with ethyl acetate (50 ml),

acidified to pH 3.0 by addition of 0.5 M H_2SO_4 and extracted with ethyl acetate (2 times 100 ml). The ethyl acetate solution was washed with a saturated solution of NaCl (2 times 100 ml), dried over $MgSO_4$ and concentrated under reduced pressure to a volume of 100 ml.

To the solution of NPS-L-Tyr–Gly (9 mmoles) in ethyl acetate (100 ml), *N*-hydroxysuccinimide (1.3 g, 11 mmoles) and dicyclohexycarbodiimide (2.1 g, 10 mmoles) were added. After 3 h at room temperature the dicyclohexylurea was removed by filtration and the filtrate was washed successively with water (150 ml), 5 % $NaHCO_3$ (100 ml), water (100 ml) and finally dried over $MgSO_4$. The clear solution was filtered and added dropwise to light petroleum (b.p. 40–60°) (100 ml). The precipitate was collected by filtration, giving 1 g of *N*-hydroxysuccinimide ester of NPS-L-Tyr–Gly (yield 23 %).

A similar procedure was used for the preparation of *N*-hydroxysuccinimide esters of NPS-L-Tyr_n–Gly ($n = 2$–3) and NPS-L-Trp–Gly.

Peptidyl-tRNA. General procedure

To a solution of *N*-hydroxysuccinimide ester of NPS-amino acid or peptide (20–30 mg) in distilled dimethylformamide (0.25 ml), 0.25 M triethanolamine (0.25 ml) of pH 8.1 was added, followed by the addition of [^{14}C]aminoacyl-tRNA (5 pmoles) dissolved in water (10 μl). The reaction mixture was shaken in a vortex test tube mixer for several min, then kept at 10° for 1 h. Cold 10 % dichloroacetic acid (0.5 ml) was added, and after 10 min at 0° cold dimethylformamide (2 ml) was added. The tRNA was isolated by centrifugation (15 000 $\times g$ for 10 min at 4°). The precipitate was washed with a mixture of acetone–diethylether (1:1, by vol.) (3 times 2 ml); the tRNA was isolated by centrifugation after each washing. Finally the precipitate was dried in a vacuum desiccator and dissolved in 0.1 M acetate buffer (pH 5.0; 0.4 ml). Then a solution of 1 M $Na_2S_2O_3$ (0.1 ml) (final concn. 0.2 M) was added and after 0.5 h at 30° 10 % dichloroacetic acid (0.5 ml) and ethanol (1 ml) were added. The tRNA was isolated by centrifugation, the precipitate washed with a mixture of acetone–diethylether (1:1, by vol.) (3 times 2 ml), dried in a vacuum desiccator and dissolved in 0.1 M acetate buffer (pH 5.0). An aliquot was treated with 0.5 M NaOH and the hydrolysate was analyzed by paper electrophoresis. The electrophoretic mobilities of the different peptides are given in Table I.

The yields of the acylations were above 98 % and the recovery of the radioactive material was between 70 and 85 %.

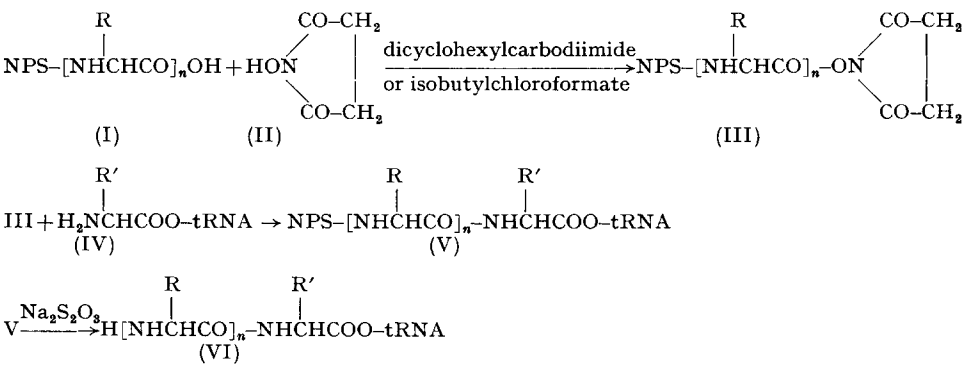
RESULTS AND DISCUSSION

The synthesis of peptidyl-tRNA containing bifunctional amino acids, as glycine or alanine, is based on the reaction between *N*-hydroxysuccinimide ester of monomethoxytrityl amino acid (or peptide) and aminoacyl-tRNA and the subsequent removal of the N-blocking group by mild acidic treatment[1–4]. In cases where the peptide which reacts with the aminoacyl-tRNA is composed of one or more trifunctional amino acids, the third functional group (–OH, $-NH_2$, –SH, or –COOH) should either be blocked during the acylation reaction or alternatively conditions have to be found in which the third functional group is not reactive.

In the case of serine and tyrosine it was found that the N-blocked carboxyl-activated amino acid could be prepared and used for acylating aminoacyl-tRNA without blocking the hydroxyl group. Thus *N*-hydroxysuccinimide ester of *N*-monomethoxytrityl-L-serine was used for the synthesis of serine-containing peptidyl-tRNA. Although the monomethoxytrityl group was used successfully as an N-blocking group in the preparation of serine as well as glycine- and alanine-containing oligopeptidyl-tRNA, it was found that in the case of *N*-hydroxysuccinimide ester of N-blocked tyrosine the yield of the acylation was low, probably because of steric hindrance. Similar steric hindrance effects were previously observed when the trityl group was used as an N-blocking group in peptide synthesis[6]. In order to overcome these difficulties, ZERVAS *et al.*[6] introduced the *o*-nitrophenylsulfenyl group (NPS) as an N-blocking group in peptide synthesis. Recently we reported on the use of NPS as an N-blocking group in the synthesis of Val-Gly-[^{14}C]Phe-tRNA.

NPS-L-serine and NPS-L-tyrosine were prepared according to the published procedure[6]. The *N*-hydroxysuccinimide esters (III) were prepared by condensing the N-blocked amino acid (or peptide) (I) with *N*-hydroxysuccinimide (II) either by using dicyclohexylcarbodiimide as condensing agent[7] or by the method of mixed anhydride developed by ANDERSON *et al*[8]. The *N*-hydroxysuccinimide ester of the NPS-amino acid (or peptide) (III) was reacted with aminoacyl-tRNA (IV) in a mixture containing 50 % dimethylformamide and 50 % aqueous solution of triethanol amine of pH 8.1 (final concn. 0.125 M). After 1 h at 10° the tRNA was precipitated and isolated by centrifugation (for details see EXPERIMENTAL), The NPS group was removed from the blocked peptidyl-tRNA (V) by $Na_2S_2O_3$ according to FORTANA *et al.*[9]. The acylation reactions were quantitative.

The reaction can be summarized in the following scheme:



A list of different oligopeptidyl-tRNA's containing serine and tyrosine which were prepared according to the above scheme is given in Table II.

The synthesis of (L-Ser)$_6$-[^{14}C]Phe-tRNA was accomplished by the use of the stepwise technique. In the first step, *N*-hydroxysuccinimide ester of NPS-L-Ser-L-Ser was reacted with [^{14}C]Phe-tRNA resulting, after removal of the N-blocking group, in the formation of (L-Ser)$_2$-[^{14}C]Phe-tRNA. The acylation reaction was repeated two more times resulting in the formation of (L-Ser)$_6$-[^{14}C]Phe-tRNA (Table III). After each acylation reaction, an aliquot was treated with alkali and the hydrolysate was analyzed by high-voltage paper electrophoresis (see Fig. 1). The hatched

TABLE II

THE SYNTHESIS OF VARIOUS OLIGOPEPTIDYL-tRNA's

N-Hydroxysuccinimide ester of NPS amino acid or peptide	*Aminoacyl-tRNA*	*Peptidyl-tRNA formed*
L-Ser	L-[^{14}C]Phe	L-Ser-L-[^{14}C]Phe*
L-Ser-L-Ser	L-[^{14}C]Phe	L-Ser-L-Ser-L-[^{14}C]Phe
L-Ser	L-[^{14}C]Ser	L-Ser-L-[^{14}C]Ser
L-Ser-L-Ser	L-[^{14}C]Ser	L-Ser-L-Ser-L-[^{14}C]Ser
L-Tyr	L-[^{14}C]Phe	L-Tyr-L-[^{14}C]Phe**
L-Tyr-Gly	L-[^{14}C]Phe	L-Tyr-Gly-L-[^{14}C]Phe
L-Tyr-L-Tyr-Gly	L-[^{14}C]Phe	L-Tyr-L-Tyr-Gly-L-[^{14}C]Phe
L-Tyr-L-Tyr-Tyr-Gly	L-[^{14}C]Phe	L-Tyr-L-Tyr-L-Tyr-Gly-L-[^{14}C]Phe
L-Trp-Gly	L-[^{14}C]Phe	L-Trp-Gly-L-[^{14}C]Phe

* Was also prepared by the reaction of the *N*-hydroxysuccinimide ester of monomethoxytrityl-L-Ser and [^{14}C]Phe-tRNA. The acylation reaction was performed as reported elsewhere[4].

** The yield of the acylation in this particular case did not exceed 77 %, probably because of steric hindrance. In all the other reactions described in this table the acylation was quantitative.

TABLE III

THE STEPWISE SYNTHESIS OF (L-Ser)$_6$-[^{14}C]Phe-tRNA

N-Hydroxysuccinimide ester of NPS peptide	*Substrate*	*Total number of steps*	*Oligopeptidyl-tRNA*
L-Ser-L-Ser	L-[^{14}C]Phe-tRNA	1	(L-Ser)$_2$-L-[^{14}C]Phe-tRNA
L-Ser-L-Ser	(L-Ser)$_2$-L-[^{14}C]Phe-tRNA	2	(L-Ser)$_4$-L-[^{14}C]Phe-tRNA
L-Ser-L-Ser	(L-Ser)$_4$-L-[^{14}C]Phe-tRNA	3	(L-Ser)$_6$-L-[^{14}C]Phe-tRNA

areas indicate the electrophoretic mobility of the synthetic markers. We did not have the appropriate markers for Ser$_4$–Phe and Ser$_6$–Phe, but from the electrophoretic mobility of the radioactive material resulting from the alkaline hydrolysate of the second and third steps compared with the known markers, it seems safe to conclude that they are Ser$_4$–Phe and Ser$_6$–Phe, respectively.

All amino acids used in this work were L-amino acids. When an N-blocked amino acid is condensed with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide, no racemization occurs. But when the same condensing agent is used in the synthesis of *N*-hydroxysuccinimide ester of N-acylpeptide, the possibility of racemization does exist. In order to overcome this difficulty, the mixed carbonic-carboxylic anhydride method was used. According to ANDERSON *et al.*[8] this method does not give rise to racemization. Another way to avoid racemization is the use of glycine at the carboxylic terminal of the peptide. Moreover, it was found that in the case of *N*-hydroxysuccinimide esters of N-blocked peptides containing tyrosine and tryptophane, the presence of the glycine at the C-terminal improves the yield of acylation. The yield of acylation of the same peptides without glycine at the C-terminal was lower, probably because of steric hindrance.

The biochemical and physical properties of the chemically-prepared oligopeptidyl-tRNA's described in this paper are at present under investigation in our laboratory.

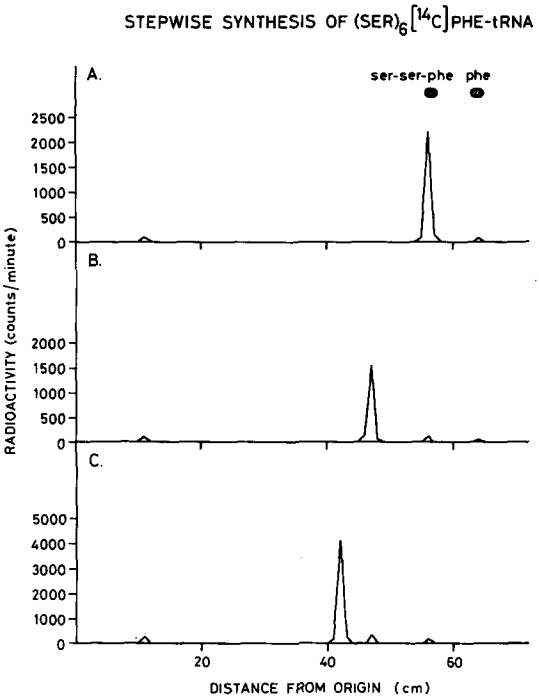


Fig. 1. Paper electrophoresis of the alkaline hydrolysates of serine-containing oligopeptidyl-tRNA. A. Alkaline hydrolysate of the reaction product of *N*-hydroxysuccinimide ester of NPS-L-Ser–L-Ser and [^{14}C]Phe-tRNA after treatment with $Na_2S_2O_3$ (first step). B. Alkaline hydrolysate of the reaction product of *N*-hydroxysuccinimide ester of NPS-L-Ser–L-Ser and L-Ser_2–[^{14}C]Phe–tRNA after treatment with $Na_2S_2O_3$ (second step). C. Alkaline hydrolysate of the reaction product of *N*-hydroxysuccinimide ester of NPS-L-Ser–L-Ser and L-Ser_4–[^{14}C]Phe–tRNA after treatment with $Na_2S_2O_3$ (third step).

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