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A SPECIFIC COLORIMETRIC AND CYTOCHEMICAL SUBSTRATE FOR RIBONUCLEASE T_2: ADENOSINE-3'-(α-NAPHTHYLPHOSPHATE)

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SUMMARY

Details are presented for the synthesis of adenosine-3'-(α-naphthylphosphate), a specific substrate for the cytochemical localization or colorimetric assay of ribonuclease T_2 (ribonucleate nucleotido-2'-transferase (cyclizing), EC 2.7.7.17) type enzymes. The synthetic route involves the preparation of 3'-*O*-acetyladenosine, which is converted stepwise to N^6-benzoyl-2',5'-di-*O*-tetrahydropyranyladenosine, followed by phosphorylation of the latter with either α-naphthylphosphoryl dichloride, or with α-naphthylphosphate in the presence of dicyclohexylcarbodiimide. Final purification of the product was attained by column chromatography on benzyl-DEAE cellulose, a procedure superior to those previously employed for purification of other nucleoside-3'-(α-naphthylphosphate) substrates. Comparative rates of hydrolysis for this substrate by ribonuclease T_2, ribonuclease T_1 (EC 2.7.7.26), pancreatic ribonuclease (EC 2.7.7.16) and phosphodiesterase II (EC 3.1.4.1) are presented and compared with those for other substrates of these enzymes. The overall findings are evaluated in terms of substrate specificities and their possible overlapping.

The synthesis and application of some α-naphthylphosphate esters of various nucleosides to the histochemical localization of phosphodiesterases and ribonucleases by the azo-dye coupling technique have already been described[1–5]. These synthetic substrates may also be employed for the colorimetric estimation of enzyme activities in homogenates and tissue extracts[6].

It appeared of interest to extend the foregoing series to substrates specific for ribonuclease T_2. Ribonuclease T_2 is a ribonucleate nucleotido-2'-transferase (cyclizing) (EC 2.7.7.17) showing no base specificity with low molecular weight substrates and only partial preference towards adenine residues in polyribonucleotides[7]. The enzyme has been isolated from *Aspergillus oryzae*, but similar types of activity have been reported in other microorganisms[8]. Plant tissues also contain ribonucleases showing no specificity with regard to the base[9]. Because of this lack of base specificity, the T_2-like ribonucleases exhibit activity towards the α-naphthyl esters of uridine and inosine[4]. The latter, however, are subject to hydrolysis also by other ribo-

nucleases[4], which render them unsuitable as specific substrates for ribonuclease T_2.

The present communication describes the preparation of adenosine-3'-(α-naphthylphosphate), which was, in fact, found to be essentially resistant to pancreatic (EC 2.7.7.16) and T_1 ribonucleases (EC 2.7.7.26), indicating that it may be employed as a specific substrate for the non-base specific T_2-type of ribonucleases. Adenosine-2',3'-cyclic monophosphate[7] and adenosine-3'-benzylphosphate[7,10] have been previously employed as specific substrates for ribonuclease T_2, but are of course not applicable to cytochemical investigations.

Adenosine-3'-(α-naphthylphosphate) was prepared essentially by the method previously described for the synthesis of other α-naphthyl esters of ribonucleoside-3'-phosphates[4], but with the introduction of some useful and important modifications. The general synthetic procedure is formulated in the Scheme on page 326.

2',5'-di-*O*-Tetrahydropyranyladenosine was obtained *via* 3'-*O*-acetyladenosine according to standard procedures[11,12]. However, phosphorylation of the 3'-OH group of the pyranylated derivative proved to be more complicated than anticipated, since the exogenous amino group readily underwent substitution in fairly high yield during phosphorylation, either with α-naphthylphosphate and dicyclohexylcarbodiimide, or with α-naphthylphosphoryl dichloride. Attempts to block the exocyclic N^6 by prolonged pyranylation[13] gave the required N^6-2'-*O*,5'-*O*-tritetrahydropyranyladenosine, but in low yield and highly impure. By contrast, benzoylation with benzoyl chloride of the amino group[14] of 2',5'-di-*O*-tetrahydropyranyladenosine provided the desired N^6-benzoyl-2',5'-di-*O*-tetrahydropyranyladenosine in good yield, and in such a form that it could be readily purified prior to phosphorylation (*Method A*), or subjected to phosphorylation directly without prior purification (*Method B*). Phosphorylation of the benzoylated derivative was carried out with α-naphthylphosphate and dicyclohexylcarbodiimide or with α-naphthylphosphoryl dichloride.

An important improvement in the overall procedure involved the replacement of the previously reported method for final purification of such substrates[4,5] from free α-naphthylphosphate and other impurities capable of liberating α-naphthylphosphate in acid medium. The acid lability of these impurities suggested that they consisted of derivatives in which the nitrogen of the heterocyclic ring was substituted by α-naphthylphosphate. Such bound α-naphthylphosphate could be removed by treatment with a mixture of anhydrous pyridine–acetic acid (1:1, by vol.), which is an established very mild reagent for hydrolysis of the P–N linkage in phosphoroimides[15]. This reagent proved quite effective inasmuch as it was without effect on the tetrahydropyranyl substituents which ensure the stability of the product in alkaline medium.

Chromatography on benzyl-DEAE cellulose proved to be the most satisfactory procedure for freeing the substrate from naphthylphosphate originating both from excess phosphorylating agent and from the above-described treatment of contaminants. It is recommended that this method supplant those previously employed for purification of other nucleoside-3'-(α-naphthylphosphate) substrates[4,5].

Table I presents the rates of hydrolysis of adenosine-3'-(α-naphthylphosphate) by ribonuclease T_2, ribonuclease T_1, pancreatic ribonuclease and phosphodiesterase II. For comparison purposes the table also exhibits the rates of hydrolysis by these enzymes of uridine-3'-(α-naphthylphosphate) and inosine-3'-(α-naphthylphosphate). Because of appreciable variations in degree of purity of the enzyme preparations,

TABLE I

RELATIVE ACTIVITIES OF VARIOUS RIBONUCLEASES AND PHOSPHODIESTERASE II AGAINST DIFFERENT SUBSTRATES

Activities of ribonuclease against RNA were estimated according to Beard and Razzel[19], using highly polymerized RNA. Phosphodiesterase II activity against thymidine-3'-(*p*-nitrophenylphosphate) was assayed as described by Razzel and Khorana[20]. Incubation in all instances, unless otherwise indicated, was for 30 min at 37 °C with 10 mM substrate and the following media: ribonuclease T_2, 0.05 M NaOH–citrate buffer, pH 4.5 and 6.3, with 3 mM EDTA; ribonuclease T_1, 0.1 M Tris–HCl buffer, pH 7.5, with 3 mM EDTA; pancreatic ribonuclease, 0.1 M Tris–HCl buffer pH 7.5; phosphodiesterase II, 0.1 M ammonium acetate buffer, pH 5.7, with 0.1% Tween 80 and 0.01 M EDTA; the naphthol liberated was assayed as described elsewhere[6]. Units of activity in all instances are in μmoles substrate hydrolyzed per hour per mg protein.

Substrate	*Ribonuclease T_2*		*Ribonuclease T_1*	*Pancreatic ribonuclease*	*Phosphodiesterase II*
	pH 4.5	*pH 6.3*			
Adenosine-3'-(α-naphthylphosphate)	42 000	52 400	0.02[1]	0.002[2]	0.20
Inosine-3'-(α-naphthylphosphate)	104 000	95 800	10	0.006[3]	0.23
Uridine-3'-(α-naphthylphosphate)	62 700	135 600	0.02[1]	5 000	0.50
RNA	17 200	190	44 320	500 000	—
Thymidine-3'-(*p*-nitrophenyl-phosphate)	—	—	—	—	160

[1] Incubation in these instances was for 5 h; these extremely low values are due to trace contaminants of ribonuclease T_2 (see text).

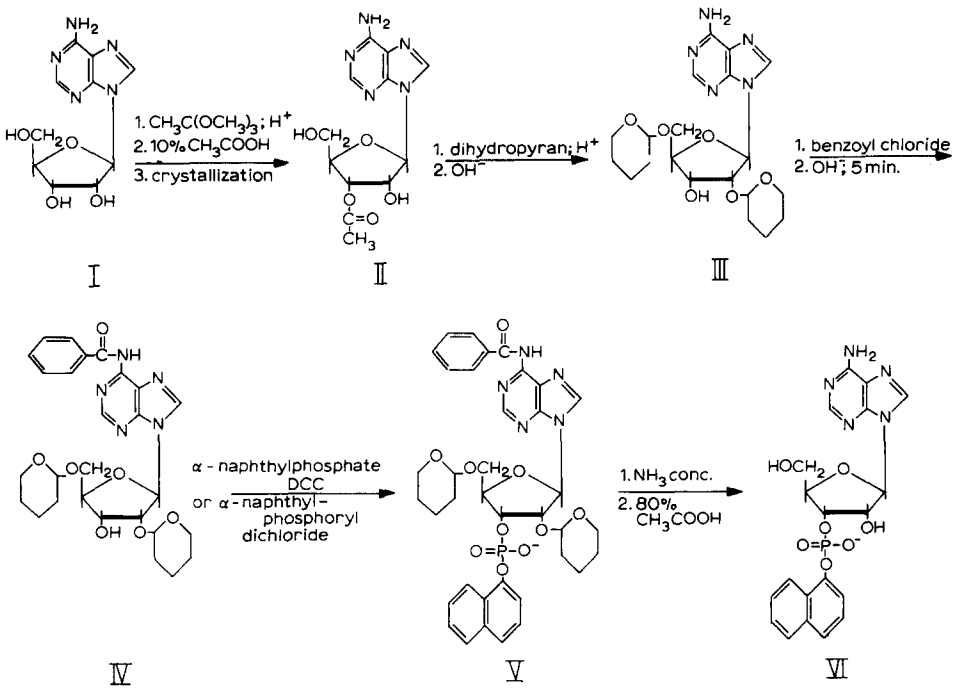
[2] Incubation for 5 h with ribonuclease A gave a value of 0.003.

[3] Incubation for 5 h with ribonuclease A gave a value of 0.005.

rendering difficult a direct comparison between the rates of hydrolysis of a given substrate, the table includes the measured activities of the enzymes against the traditional (but non-specific) substrate RNA for the ribonucleases, and thymidine-3'-(*p*-nitrophenylphosphate) for phosphodiesterase II.

From Table I it is clear that adenosine-3'-(α-naphthylphosphate) is quite resistant to ribonuclease T_1. The value of $\leqslant$0.02 μmole/h/mg protein for the rate of hydrolysis of this substrate by ribonuclease T_1 is an upper limit observed with a large excess of some commercial preparations; with other preparations the value was below the level of detection. An excess of enzyme was employed also in the case of pancreatic ribonuclease, showing the substrate to be essentially inert to this enzyme. Furthermore, adenosine-3'-(α-naphthylphosphate) was only slightly susceptible to phosphodiesterase II.

The rate of hydrolysis of the substrate by ribonuclease T_2 was examined at pH 4.5, which is optimal for the rate of hydrolysis of high molecular weight RNA; and at pH 6.3, optimal for the hydrolysis of low-molecular weight substrates[7]. At pH 4.5, enzyme activity is maximal against inosine-3'-(α-naphthylphosphate), and at pH 6.3 against uridine-3'-(α-naphthylphosphate). These results are in qualitative agreement with the observations of Sato *et al.*[7] and Imazawa *et al.*[8], who demonstrated the maximal rate of hydrolysis of uridine-2',3'-cyclic monophosphate and the dinucleoside monophosphates UpX (where X= U, C, G, A) by ribonuclease T_2 at pH 5.5.



Notwithstanding that adenosine-3′-(α-naphthylphosphate) is hydrolyzed by ribonuclease T_2 at a lower rate than the corresponding inosine and uridine substrates (see Table I), its specificity makes it the substrate of choice both for histochemical purposes, as well as for colorimetric estimations of activity in cellular extracts. Furthermore, its rate of hydrolysis by ribonuclease T_2 is 2.5-fold that for hydrolysis of RNA, testifying to its higher sensitivity as a substrate.

Of additional interest is the fact that the rate of hydrolysis of adenosine-3′-(α-naphthylphosphate) by ribonuclease T_2 exceeds that for adenosine-3′-benzylphosphate. From the data of Molemans *et al.*[10] for the rate of hydrolysis of the latter substrate, it may be readily calculated that adenosine-3′-(α-naphthylphosphate) is hydrolyzed at pH 4.5 at a rate approximately 90-fold higher than the corresponding benzyl substrate. This is in agreement with the previously observed 80-fold higher rate of hydrolysis of uridine-3′-(α-naphthylphosphate), relative to uridine-3′-benzylphosphate, by pancreatic ribonuclease (Witzel, A., personal communication), a phenomenon ascribed to the higher leaving tendency of the α-naphthyl group[2].

From Table I the rate of hydrolysis by phosphodiesterase II of adenosine-3′-(α-naphthylphosphate), 0.20, is 800-fold lower than that for the optimal synthetic substrate, thymidine-3′-(p-nitrophenylphosphate), and is consequently negligible with the normally encountered ratios of ribonuclease T_2 to phosphodiesterase II. In the event that one were to come across a cell in which the ratio of ribonuclease T_2 to phosphodiesterase II is considerably lower, it would likely prove possible to eliminate substrate susceptibility to phosphodiesterase II by the introduction of a 5′-*O*-benzyl substituent. This has already been shown to render uridine-3′-(α-naph-

thylphosphate) completely resistant to phosphodiesterase II, with only a small reduction in susceptibility to pancreatic ribonuclease[5].

The very slight susceptibility of adenosine-3'-(α-napthylphosphate) to pancreatic ribonuclease calls for some comment. It had previously been noted by Beers[16], and subsequently confirmed by Imura *et al.*[17], that poly(A) and poly(I) are slowly hydrolyzed by elevated concentrations of pancreatic ribonuclease, the products of hydrolysis being adenosine 2',3'-cyclic monophosphate and adenosine 3'-monophosphate. It will be noted from Table I that inosine-3'-(α-naphthylphosphate) likewise exhibits slight susceptibility to pancreatic ribonuclease. That this susceptibility is associated with pancreatic ribonuclease, and not with some contaminating enzyme, was shown by the fact that similar low rates of hydrolysis of both these substrates were observed when pancreatic ribonuclease was replaced by ribonuclease A (Table I). This was further supported by the fact that no detectable activity could be observed on submission of thymidine-3'-(α-naphthylphosphate) to pancreatic ribonuclease under the same conditions.

Trace hydrolysis of adenosine-3'-(α-naphthylphosphate) by excess ribonuclease T_1 was found to be non-reproducible and therefore suggestive of contamination of some batches of ribonuclease T_1. Since the experimental value for hydrolysis of uridine-3'-(α-naphthylphosphate) with these batches of ribonuclease T_1 was approximately equal to that for adenosine-3'-(α-naphthylphosphate), it appears that hydrolysis of the latter could not have been a consequence of deamination due to adenosine deaminase contamination of the ribonuclease T_1. The possibility of hydrolysis of these substrates by phosphodiesterase II contaminants in ribonuclease T_1 was eliminated by demonstrating that thymidine-3'-(p-nitrophenylphosphate) was not affected. It therefore follows that the very low hydrolysis of the 3'-(α-naphthylphosphate)s of adenosine and uridine by some batches of ribonuclease T_1 is due to trace contaminants of ribonuclease T_2.

Finally, direct tests demonstrated that adenosine-3'-(α-naphthylphosphate) was not hydrolyzed either by homogenates, or fixed and unfixed sections, of kidney, spleen, pancreas and liver of the rat, confirming that mammalian tissues are devoid of ribonuclease T_2-type enzymes. By contrast, the substrate has proven useful in studies on the localization of the ribonucleases of wheat shoots[18]. It should also be of use in the screening of microorganisms for ribonuclease T_2-type of activity, as well as in enzyme purification studies and investigations of kinetics.

EXPERIMENTAL

Whatman paper No. 1 was used for ascending paper chromatography. The solvent systems employed were: (A) *n*-butanol–water (86:14, v/v), and (B) isopropanol–conc. NH_4OH–water (7:1:2, v/v/v).

The time-course of the synthetic reactions was followed by thin-layer chromatography, using microscope slides coated with Merck TLC silica gel GF_{254}. The chromatograms were developed with chloroform–methanol (9:1, v/v, Solvent C).

The chloroform employed for column chromatography was first extracted with water, dried over $CaCl_2$, and then subjected to redistillation. The silicic acid used for column chromatography was Mallinckrodt type CC7. Benzyl-DEAE-cellulose (a Sigma product), carbonate form, and triethylammonium bicarbonate buffer

were prepared as described by Khorana and Connors[21]. The procedure of Friedman and Seligman[22] was used for the preparation of α-naphthylphosphoryl dichloride and α-naphthylphosphoric acid.

High molecular weight yeast ribosomal RNA was supplied by the Department of Molecular Biology, Łodz Medical School. Crystalline pancreatic ribonuclease was a Reanal (Budapest, Hungary) product, stabilized in solution with 0.1% gelatine. Ribonuclease A was a Sigma product. Crystalline ribonuclease T_1 and ribonuclease T_2 were purchased from Sankyo (Tokyo, Japan). Phosphodiesterase II, a Worthington (Freehold, N.J., U.S.A.) preparation, was used in all cases except with uridine-3'-(α-naphthylphosphate) for which pancreatic ribonuclease-free phosphodiesterase II (purified and supplied by Dr G. Bernardi) was employed. Snake venom phosphodiesterase (EC 3.1.4.1) and bacterial alkaline phosphatase (EC 3.1.3.1), purity grade BAPF, were Worthington preparations.

3'-O-Acetyladenosine (II) was prepared according to the procedure of Fromageot *et al.*[11], as already applied to 3'-*O*-acetylinosine[4] with minor modifications. A suspension of adenosine (9 g, 34 mmoles) in 27 ml anhydrous dimethylformamide was treated with trimethylorthoacetate (54 ml) and trifluoroacetic acid (2.5 ml) for 2 h. After work-up[4], the mixture was hydrolyzed in 10% acetic acid and the whole brought to dryness under reduced pressure. The residue was dried by repeated evaporation with ethanol–benzene. The product was finally purified by elution with chloroform containing 4% methanol from a 25 cm × 2.5 cm column of silicic acid, and crystallized from anhydrous ethanol to yield 5.2 g (50%), m.p. 180–181 °C, and R_F values of 0.42 and 0.20 with solvents A and C, respectively, as compared to 0.22 and 0.04 for adenosine.

2',5'-di-O-Tetrahydropyranyladenosine (III) was obtained essentially as described for 2',5'-di-*O*-tetrahydropyranylinosine[4] with the exception that dimethylformamide was omitted from the reaction mixture and the ether-extraction step omitted in the work-up procedure.

3'-*O*-Acetyladenosine (2.6 g, 8.4 mmoles), toluene-*p*-sulfonic acid monohydrate (1.77 g) and dihydropyran (4.3 ml) were stirred magnetically in anhydrous dioxane (26 ml) under anhydrous conditions for about 2 h to give a clear solution. After work-up the residue was deacetylated with methanol–conc. NH_4OH (1:1, v/v), dried over P_2O_5 and chromatographed on a 25 cm × 2.5 cm silicic acid column with 2% methanol in chloroform to yield 2.8 g (78%) of purified product, which gave a single spot with Solvent A (R_F 0.89). On thin-layer chromatography with Solvent C, diastereoisomers were resolved with R_F values of 0.52 and 0.58 in a ratio of approx. 1:1.

Method A (with prior purification of blocked nucleoside)

N^6-Benzoyl-2',5'-di-O-tetrahydropyranyladenosine (IV) was prepared as follows: 2',5'-di-*O*-Tetrahydropyranyladenosine (1.6 g, 3.6 mmoles) was dissolved in 12 ml anhydrous pyridine and to this solution 3 ml of freshly distilled benzoyl chloride were added. After 2 h at room temperature, the solution was poured into 200 ml chilled water and the mixture extracted with chloroform. The organic phase was brought to dryness, the residue dissolved in 12 ml ethanol and to this solution was added 8.5 ml pyridine, followed by 33 ml of a mixture of ethanol–2 M NaOH (1:1, v/v). The mixture was left for 5 min at room temperature, after which 12 g of Dowex

50W-X8 (pyridinium) was added. The resin was then removed by filtration, the filtrate brought to dryness and the residue dried under vacuum over P_2O_5 and dissolved in 15 ml chloroform. The solution was filtered and deposited on a 25 cm × 3 cm column of silicic acid, which was washed with chloroform. The product was then eluted with chloroform containing 1% methanol. The appropriate fractions were brought to dryness under vacuum yielding a pale yellow glass which was dried over P_2O_5. Yield, 1.34 g (66%), R_F in Solvent A, 0.90. Thin-layer chromatography with Solvent C resolved diastereoisomers with R_F values 0.70 and 0.78.

2′,5′-di-O-Tetrahydropyranyladenosine-3′-(α-naphthylphosphate) (V) was prepared as follows. To a solution of α-naphthylphosphoric acid (1.62 g, 8.4 mmoles) in 15 ml anhydrous pyridine were added 730 mg (1.4 mmoles) of N^6-benzoyl-2′,5′-di-*O*-tetrahydropyranyladenosine. To ensure anhydrous conditions, the solution was brought to dryness, the residue taken up in anhydrous pyridine and again brought to dryness; this was repeated twice and the residue finally taken up in 25 ml dry pyridine. Dicyclohexylcarbodiimide (2.5 g, 1.21 mmoles) was added next and the reaction vessel sealed and kept in the dark for 24 h at room temperature. The reaction mixture was then immersed in solid CO_2–methanol with 5 ml 50% aqueous pyridine added, and the mixture again brought to room temperature and left for 1 h. It was then brought to dryness and the residue dissolved in 50 ml 9 M NH_4OH and left overnight. The solution was again brought to dryness and the residue dissolved in 50 ml water, which was then extracted with ether; the aqueous phase was brought to dryness and the residue dried overnight *in vacuo* over P_2O_5. The dried residue was next dissolved in 5 ml anhydrous pyridine, to which was added 5 ml anhydrous acetic acid, and the solution stored at 45 °C for 3 days. The solution was then poured into 500 ml ice water, rapidly brought to pH 9 with conc. NH_4OH, and applied to a 60 cm × 3 cm column of benzyl-DEAE cellulose (carbonate form). The column was washed with 30% ethanol, following which the title compound was eluted with 0.026 M triethylammonium bicarbonate in 30% ethanol. The appropriate fractions were collected, pooled, brought to dryness, the residue taken up in water, brought to dryness several times, and finally lyophilized to remove traces of triethylammonium bicarbonate. Yield of product was 60%, with R_F values in Solvents A and B of 0.65 and 0.90, respectively.

Method B (simplified procedure)

137 mg of 2′,5′-di-O-tetrahydropyranyladenosine (III) was benzoylated as above, but with omission of the column chromatography on silicic acid. The crude, dried product was dissolved in 5 ml anhydrous pyridine and to this was added, dropwise, 0.24 ml α-naphthylphosphoryl dichloride over a period of 30 min. The solution was stored for 24 h, cooled in solid CO_2–methanol with 0.5 ml 50% aqueous pyridine added, after which the mixture was brought to dryness. The residue was dissolved in 80 ml chloroform and was then extracted with 10 ml water, after which the organic phase was brought to dryness. The residue was taken up in 20 ml 9 M NH_4OH and left overnight. Further work-up was as described in Method A, above, with an overall yield of 33%. The lyophilized product was stored over P_2O_5, under which conditions it is stable.

The di-O-tetrahydropyranylated substrate is stable as such and may be stored indefinitely under anhydrous conditions in a dessicator. Prior to use as a substrate,

the tetrahydropyranyl protecting groups are removed by treatment with 80% acetic acid as described elsewhere[4]. It should be noted that at pH values above 8 the substrate is itself labile, due to the high leaving tendency of the α-naphthyl group[2].

The homogeneity of the substrate was attested to by chromatography with various solvent systems, and its complete resistance to *Escherichia coli* alkaline phosphatase and snake venom phosphodiesterase, demonstrating the absence of free α-naphthylphosphate or the isomeric adenosine-5′-(α-naphthylphosphate). On incubation with an excess of ribonuclease T_2, followed by paper chromatography of the products of hydrolysis, quantitative conversion was observed to adenosine-3′-phosphate and free α-naphthol, with R_F values of 0.04 and 0.95 in Solvent A.

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