Method for the Selective 2′-O-Substitution of Nucleosides

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This paper presents a new method for selective reactions of predetermined sugar hydroxyls of nucleosides. Succinylated nucleosides were investigated as examples for the use of the cyclic phosphate group for protecting purposes. Starting from cyclic AMP the 2′-O-group was selectively succinylated yielding 93% 2′-O-succinyl cyclic AMP. The cyclic phosphate was enzymatically dephosphorylated in a one step procedure under neutral conditions and 2′-O-succinyl adenosine containing a small amount of the 3′-O-isomer was produced in 91% yield. When establishment of equilibrium of the 2′-O- and 3′-O-isomers was allowed, 54% yield of crystallized 3′-O-succinyl adenosine was produced. The results suggest that the easily accessible cyclic monophosphates are good protecting groups for the production of nucleoside derivatives, especially at the 2′-O-position under neutral conditions.

Introduction

The increasing interest for the nucleosides has initiated the search for new and selective reactions of predeterminated sugar hydroxyls. Fromageot *et al.* [1] reacted 2′-O- and 3′-O-hydroxyls with trimethylorthoester and obtained a mixture of 2′-O- and 3′-O-esters after acidic hydrolysis. Wagner *et al.* [2] employed 2′,3′-O-dibutylstannylene in place of the orthoester. Tritylethers and their derivatives, butyldimethylsilyl- and tetrahydropyranoylethers have been employed as protecting groups for selective sugar substitution [3], all requiring acidic or basic cleavage. In this study a commercially available product with a cyclic monophosphate as protecting group was used. Cleavage of the protecting group did not require basic or acidic conditions. Adenosine 3′,5′-cyclic monophosphate (cyclic AMP) was succinylated in the 2′-O-position and 2′-O-succinyl cyclic AMP enzymatically dephosphorylated to give 2′-O-succinyl adenosine.

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Materials and Methods

Chemicals

Cyclic AMP, 5′-adenosine monophosphate (5′AMP), adenine, hypoxanthine, adenosine, inosine, phosphodiesterase (PDE) from beef heart and alkaline phosphatase from calf intestinal mucosa purity I were purchased from Boehringer Mannheim. Crotalus atrox venom of the western diamondback rattlesnake containing 5′-nucleotidase, N^6-butyryl cyclic AMP, N^6,2′-O-dibutyryl cyclic AMP and an authentic sample of 2′-O-succinyl cyclic AMP were purchased from Sigma Chemie, München. All other reagents used were analytical grade.

Thin-layer chromatography

Reaction products were checked by thin layer chromatography on plates coated with cellulose and fluorescence indicator CEF from Riedel de Haen, Hannover and silica gel plates 60 F 254 from Merck, Darmstadt. Thin-layer chromatography was performed in several solvent systems. System A: 2-butanol/methanol/ethylacetate/acetic acid/H_2O (7 : 3 : 4 : 2 : 4), pH 2.8; system B: saturated ammonium sulfate/1 M sodium acetate/isopropanol (80:13:3), pH 7.0; system C: amylalcohol/formic acid/H_2O (3 : 2 : 1); system D: ethanol/0.5 M ammonium acetate (5 : 2); system E: 1-butanol/methanol/ethylacetate/25% ammonium hydroxide (7:3:4:4); system F: isobutyric acid/2 M ammonium hydroxide (66:34); system G: 1-butanol/ethanol/0.25 M ammonium acetate (3 : 5 : 2).

Instrumentation

UV spectra were recorded on a Zeiss PMQ II or an automatic DMR 22 spectrometer. Elution diagrams of column chromatography were taken whenever available with LKB uvicord UV meters at 254 nm and 280 nm. Mass spectra were recorded on types MS 9 and CH 4 of AEI, Manchester/England. Varian XL-150, Jeol INM-C-60-HL and Bruker 90 spectrometers were used for the pmr spectra. $Me_3Si(CD_2)_2COO_2$ Na or TMS were taken for reference.

Phosphate determination

Inorganic phosphate was measured according to Fiske and Subbarow [4].

Results

Preparation of 2'-O-succinyl cyclic AMP

Starting from a procedure reported by Cailla *et al.* [5] for succinylation of cyclic AMP in micro assays in water free medium a synthesis of 2'-O-succinyl cyclic AMP in preparative scale was performed. 250 mg cyclic AMP and 1.125 g succinyl anhydride were dissolved in 25 ml water and 2.5 ml triethylamine and shaken at room temperature for one hour. Lyophilization and solution in 25 ml water were repeated three times to remove the triethylamine. Lyophilization did not affect 2'-O-succinyl cyclic AMP. The last lyophilizate was dissolved in 10 ml water and applied to a 30 × 2.5 cm QAE-Sephadex A-25 column. 50 fractions were first eluted with 500 ml 250 mM ammonium acetate, pH 6, within 5 h. At this concentration of the buffer cyclic AMP was eluted. 2'-O-succinyl cyclic AMP was eluted with 900 ml 500 mM ammonium acetate, pH 6, for 10 h and in 100 fractions. The yield of 2'-O-succinyl cyclic AMP relative to the starting material was 93%.

Preparation of 2'-O-succinyl adenosine

19.2 mg 2'-O-succinyl cyclic AMP (44.7 µmol) was dissolved in 5.5 ml 30 mM phosphate buffer, pH 7 and 3.5 ml 10 mM $MgCl_2$. The pH of the solution was adjusted to 6.85 by 1 N NaOH. 0.8 mg phosphodiesterase in a volume of 0.8 ml and 0.5 mg alkaline phosphatase in a volume of 0.5 ml were added without raising the pH over 6.9. The solution was incubated in a beaker in a shaking waterbath at 37 °C. After 80 min reaction time no nucleosides other than 2'-O-succinyl adenosine could be detected chromatographically in the incubate. The total incubate of 11.3 ml was applied to a 20 × 2 cm QAE-Sephadex A-25 column. The enzymes were washed off the column with 700 ml water in 11 h. The nucleosides were eluted with 900 ml 0.3 M ammonium acetate buffer, pH 6.5 in 120 fractions à 7.5 ml in 8 h. 2'-O-succinyl adenosine showed an elution peak (97.5% yield) with a maximum at fraction 51. Succinyl inosine (< 0.5 yield) gave a peak at fraction 42.

Measures to reduce the equilibration of 2'-O-succinyl adenosine and 3'-O-succinyl adenosine were operation of the column in the cold room at 4 °C and immediate freezing of the eluted succinyl adenosine followed by lyophilization. Under optimum conditions 2'-O-succinyl adenosine was isolated containing no pmr detectable 3'-O-succinyl adenosine.

Preparation of 2'(3')-O-succinyl adenosine

21.2 mg 2'-O-succinyl cyclic AMP was dissolved in 4 ml 10 mM $MgCl_2$ and 5 ml 30 mM phosphate buffer, pH 8.0. The pH of the solution was adjusted to 8.0 by addition of 1 N NaOH. 50 µg phosphodiesterase and 50 µg alkaline phosphatase were added.

Table I. UV data and R_F-values of nucleosides, nucleotides and their succinyl derivatives.

	max at pH 6	Cellulose system A	Cellulose system B	Cellulose system C	Cellulose system D	Silicagel system E	Cellulose system F	Cellulose system G
2'-O-succinyl adenosine	259	0.61	0.32	0.52	0.48	0.38	0.72	
2'-O-succinyl inosine	246	0.54			0.45	0.19	0.62	
adenosine	258	0.51	0.45	0.62	0.50	0.49	0.81	0.58
inosine	245	0.29	0.19		0.48	0.24	0.42	0.47
2'-O-succinyl cyclic AMP	259	0.39	0.13	0.34	0.16	0.44	0.62	
cyclic AMP	258	0.23	0.13	0.46	0.45	0.29	0.56	0.23
2'-O-succinyl 5'AMP	259	0.11			0.02	0.05		
5'AMP	258	0.06			0.05	0.05		0.05
adenine		0.54			0.52		0.95	0.70
hypoxanthine	247	0.48			0.48			0.52
3',5'-O-succinyl adenosine	260	0.76			0.23			
2',5'-O-succinyl adenosine	260	0.76			0.25			

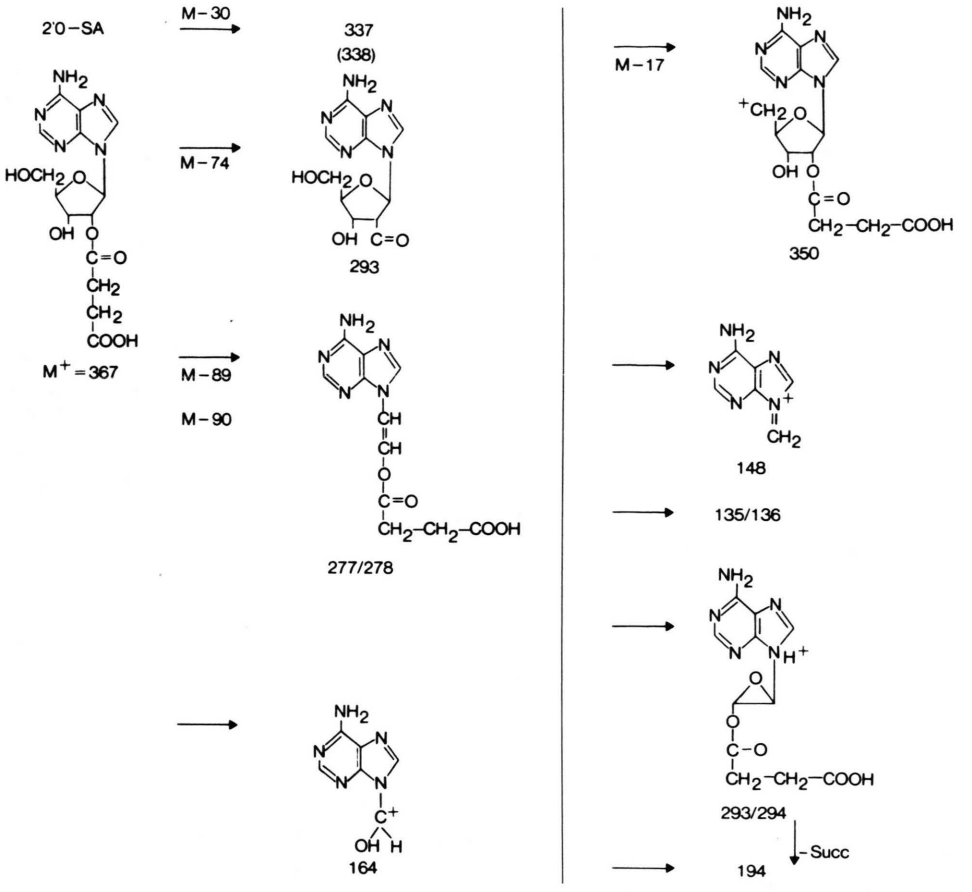

Fig. 1. Degradation pattern of 2'-O-succinyl adenosine in the mass spectrometer.

After 54 h at 37 °C the reaction was stopped by addition of 1 ml 55% TCA. The solution was centrifuged for 2 min at 12000 × *g* and the pellet was washed. TCA was extracted with 3 washes of ethylether and the remaining ether evaporated by a nitrogen stream. The solution was titrated to pH 7 with 1 N NaOH and applied on a 30 × 2 cm QAE-Sephadex A-25 column. The column was eluted in 223 fractions with 2 l of a 300 mM ammonium acetate buffer, pH 7 containing 30 mM phosphate of pH 7. Adenosine was eluted with 18% yield in fractions 27–30, 2'(3')-O-succinyl adenosine with 79% yield in fractions 91–96 which consisted of about two thirds 3'-O-succinyl adenosine as indicated by pmr. A 54% over all yield crystallized from aqueos ethanol containing 3'-O-succinyl adenosine.

Structure determination

UV data and R_F values are shown in Table I. Pmr is applicable to distinguish between 2' and 3' isomers because of the downfield shift of the H-1' caused by *cis* substituents at the C-2' position [6]. The chemical shift $\delta = 6.15$ of the H_1 was attributed to 2'-O substitution by comparison of data of 2'(3')-O acetyl

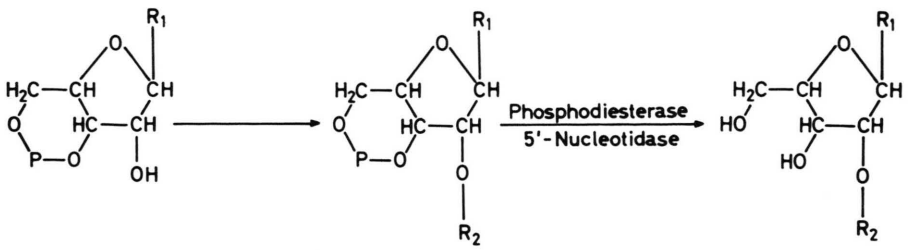

Fig. 2. Generalized two-step procedure for the synthesis of 2′-O-substituted nucleosides.

adenosine mixtures [7], of stable 2′-AMP and 3′-AMP and of succinyl adenosine isomer mixtures prepared by another method [8].

The mass-spectrum of 2′-O-succinyl adenosine showed peaks at *m/e* (relative intensity): 293 (5%), 278 (1%), 250 (20%), 194 (3%), 177 (26%), 163 (66%), 148 (15%), 136 (80%), 135 (100%). The peak at *m/e* = 278 corresponds not with the degradation pattern of 3′-O-succinyl adenosine and 5′-O-succinyl adenosine (Fig. 1).

Discussion

The procedure for 2′-O-acylation using an anhydride of a dicarbonic aliphatic acid was improved in this study. Cleavage of the 2′-O-alkylated or acylated 3′: 5′-cyclic nucleotides to give 5′-nucleotides using a phosphodiesterase from beef heart and degradation of 5′-nucleotides to give 2′-O-nucleosides using alkaline phosphatase or nucleotidase can be easily accomplished in one single step if the conditions concerning possible byproducts are carefully regarded. Byproducts may result from hydrolysis if the reaction is run at alkaline pH in the pH optimum of the enzymes employed. Deamination may be a problem if alkaline phosphatase contains deaminase being negligible at the pH optimum but producing byproducts at pH 6.

Stable ethers and stable inorganic esters including stable 2′-O-toluene sulfonic esters, but no stable acyl esters substituted at positions 2′-O and 3′-O or nucleosides have been reported. Few acyl esters have been described: formyl-, acetyl- and some aroylesters, all undergoing acyl migration [7, 9, 10]. The few available data of acyl migration of acyl esters supported by our own results led to the following statements about isomerisation: (1) The 3′-isomer prevailed over the 2′-isomer in the proportion 2 : 1 under equilibrium conditions. (2) The equilibrium constant is obviously only determined by the structure and not by the pH value. (3) Only 3′-O-isomers have been crystallized from 2′-O $\rightleftharpoons$ 3′-O-mixtures. (4) The rate constant of equilibrium increases in alkaline solution directly proportional to the pH starting from pH 7.5 [11]. (5) The rate of hydrolysis is very much slower than the isomerisation rate. The consequence for the preparation of 2′-O-succinyl adenosine has been to work under conditions slowing down isomerisation. Hydrolysis was no real problem under those conditions.

The procedure given for the synthesis of 2′-O-succinyl adenosine is especially suitable for the synthesis of the isomerisable 2′-acylisomers for the purpose of pmr studies. The procedure given for the synthesis of crystalline 3′-O-succinyl adenosine is especially suitable for the synthesis of equilibrium mixtures, for the crystallization of the prevailing isomer, and for the synthesis of stable 2′-compounds not subject to isomerisation or hydrolysis.

A cyclic phosphate group has been employed in this paper as a protection group (Fig. 2), an aspect under which cyclic phosphates have not been looked upon so far. The cyclic phosphate as a protection group bears the following advantages: (1) blockade of two adjacent hydroxyls (3′ and 5′), (2) activation of the remaining 2′ O-position by means of the electron withdrawing phosphate, (3) selective removal by enzymes, thus avoiding the addition of acids or bases.

The known procedures for selective sugar substitution employing orthoesters, dibutylstannylene, organic or silyl ethers do not show any of the advantages mentioned. The fact that the removal of the cyclic phosphate takes place under neutral conditions favours the use of this protection group especially for the synthesis of hydrolysable products such as 2′-O-acyl nucleosides.

The synthesis employing cyclic phosphate as protecting group did work with such a sensitive compound as 2′-O-succinyl adenosine. The stable derivatives of nucleosides such as esters of inorganic acids, 2′-O-alkylnucleosides and other analogues should be even easier to be synthesized. An additional advantage is that many cyclic phosphates as starting products are commercially available or conveniently synthesized [12, 13].

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