Nucleic Acids. 13. 3′-O- and 2′-O-Esters of 1-β-D-Arabinofuranosylcytosine as Antileukemic and Immunosuppressive Agents[1]

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2′-O- and 3′-O-esters of ara-cytidine were prepared, incorporating the palmitoyl, stearoyl, and benzoyl radicals. These compounds were tested for their immunosuppressive and antileukemic activity in mice in comparison with previously tested 5′-O-esters. Most of the esters showed a low order of activity by these evaluations. No 2′-O- or 3′-O-ester was superior to its corresponding 5′-O-ester, although the 3′-O-stearate was only slightly less effective than its 5′-O counterpart in antileukemic activity. The 2′-O,3′-O-diesters were inactive or of a low order of activity in both tests.

The 5′-esters of 1-β-D-arabinofuranosylcytosine with adamantane-1-carboxylic acid[2] and a wide variety of other acids[3] have been prepared, and several of these derivatives have shown therapeutic activities superior to the unsubstituted 1-β-D-arabinofuranosylcytosine (Cytosar, cytosine arabinoside, cytarabine, ara-cytidine, ara-C) in the treatment of L1210 leukemic mice or suppression of the immune response in mice[4] or rats.[5,6] It was important to determine whether these superior activities were unique for the 5′-esters of ara-C. Some preliminary tests with a substituted 3′-ester of ara-C, the 5′-O-trityl-3′-O,N^4-dibenzoyl-ara-cytidine, had shown moderate immunosuppressive activity in the graft *vs.* host reaction.† Consequently, the synthesis of some 2′-O- and 3′-O-esters of ara-C has been undertaken.

The synthetic methods used have in general produced mixed products containing 2′- and 3′-monoesters as well as 2′,3′-diesters. The separation of 2′-esters from 3′-esters is a tedious process. Consequently, the acyl and aryl derivatives which had shown the best therapeutic activity in the 5′-ester series[3] were selected for the initial evaluation of 2′- and 3′-esters. These included 2′-O- and 3′-O-palmitoyl esters (**6a** and **5a**) and 2′-O- and 3′-O-stearoyl esters (**6b** and **5b**) of the aliphatic acid series, and 2′-O- and 3′-O-benzoyl esters (**6c** and **5c**) of the aromatic acid series. In each instance, 2′-O,3′-O-diesters (**7**) were obtained as by-products of the desired monoester preparation, and these diesters were also purified and tested for antileukemic or immunosuppressive activity.

This work was completed before the paper by Nikolenko, *et al.*,[7] appeared describing 2′- and 3′-O-benzoyl derivatives of adenosine. As a starting material for their synthesis they used unsubstituted adenosine and carried out the benzoylation in a mixed solvent system containing water. Under these conditions benzoylation of the 5′-O-hydroxyl and amino group of adenine also occurs. As a starting material for our synthesis of 2′-O- and 3′-O-esters of ara-C, the 5′-O-trityl-N^4-(β,β,β-trichloroethoxycarbonyl)-ara-C (**1**) described by Gish, *et al.*,[3] was used to prevent 5′-O- and N^4-acylation (see Scheme I). This intermediate was dissolved in anhydrous pyridine and reacted with an approximately equimolar quantity of the appropriate acid anhydride or acid chloride at room temperature. Even with this limited quantity of acylating reagent, the reaction mixture still contained a considerable quantity of the 2′-O,3′-O-diester of 5′-O-trityl-N^4-(β,β,β-trichloroethoxycarbonyl)-ara-C (**4**) in addition to the corresponding 2′- and 3′-monoesters (**3** and **2**) and some unreacted starting material (**1**). Separation of the components was accomplished at the fully protected stage by chromatography on silica gel. The purified fractions were then subjected to various appropriate conditions to selectively remove the 5′-O-trityl residue[3] and the N^4-(β,β,β-trichloroethoxycarbonyl) group.[8] The resulting 2′-O- or 3′-O-monoesters of ara-C (**6** and **5**) or the corresponding 2′-O,3′-O-diesters of ara-C (**7**) were then further purified by recrystallization. The yields of the purified 2′-O- and 3′-O-monoesters of ara-C were in the range of 2–5%, while the yield of 2′-O,3′-O-diester was about 18% in the dipalmitoyl case. Assignments of structure for the monoesters was based on nmr.

Biological Activity. The immunosuppressive activities of the 2′- and 3′-esters of ara-C were compared with those of the 5′-esters using the hemagglutinin response of mice to sheep erythrocytes. The 2′-esters and 3′-esters were somewhat less active than 5′-esters under all test conditions. The 2′,3′-diesters were inactive under similar conditions.

Antitumor activities (L1210 leukemic mice) of the 2′- and 3′-esters and 2′, 3′-diesters of ara-C are shown in Table III. The best activity for a monoester in this series was obtained in the case of the 3′-O-stearyl derivative of ara-C (**5b**) with a life-span increase of about 300%. Of the 2′-derivatives tested, only the 2′-stearate (**6b**) showed significant (but low) antitumor activity. Only the 3′-O-stearyl derivative (**5b**) approached the activity previously obtained for the 5′-O-esters[4-6] in the same test system (Table III). All of the 2′,3′-diesters tested were of a low order of activity or inactive in these tests.

Experimental Section‡

Synthesis of 2′- and 3′-Monoesters and 2′,3′-Diesters of Ara-C from 5′-O-Trityl-N^4-(β,β,β-trichloroethoxycarbonyl)cytosine Arabinoside. 5′-O-Trityl-N^4-(β,β,β-trichloroethoxycarbonyl)-ara-C (19.8 g, 30 mmoles) was dissolved in 150 ml of anhyd pyridine, and the appropriate acid anhydride or acid chloride (33 mmoles) was added at room temp. The mixture was stirred over night. The resulting clear soln was concd *in vacuo*, and the residual syrup was taken up in EtOAc, concd again to remove pyridine, and redissolved in Et_2O. The Et_2O was extd with $NaHCO_3$ soln and again evapd to a syrup. The syrup was fractionated by chromatography on silica gel, using EtOAc as the eluent and following the elution by tlc. The first fractions contained the substituted 2′,3′-diester ara-C but the latter fractions were mixtures which contained the diester and both monoesters. Rechromatography using various proportions of EtOAc and cyclohexane sepd the diester and also eluted the substituted 3′-O-ester and 2′-O-ester in that order. When large quantities of material were run, a small yield of product traveling between the 3′-O-ester and the 2′-O-ester was sometimes obtained. Upon the use of procedures for removal of the 5′-O-trityl and N^4-trichloroethoxycarbonyl groups, this intermediate spot yielded the 3′-O-ester of

†G. D. Gray, W. J. Wechter, and H. A. Karnes, unpublished data.

‡Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical value.

Scheme I

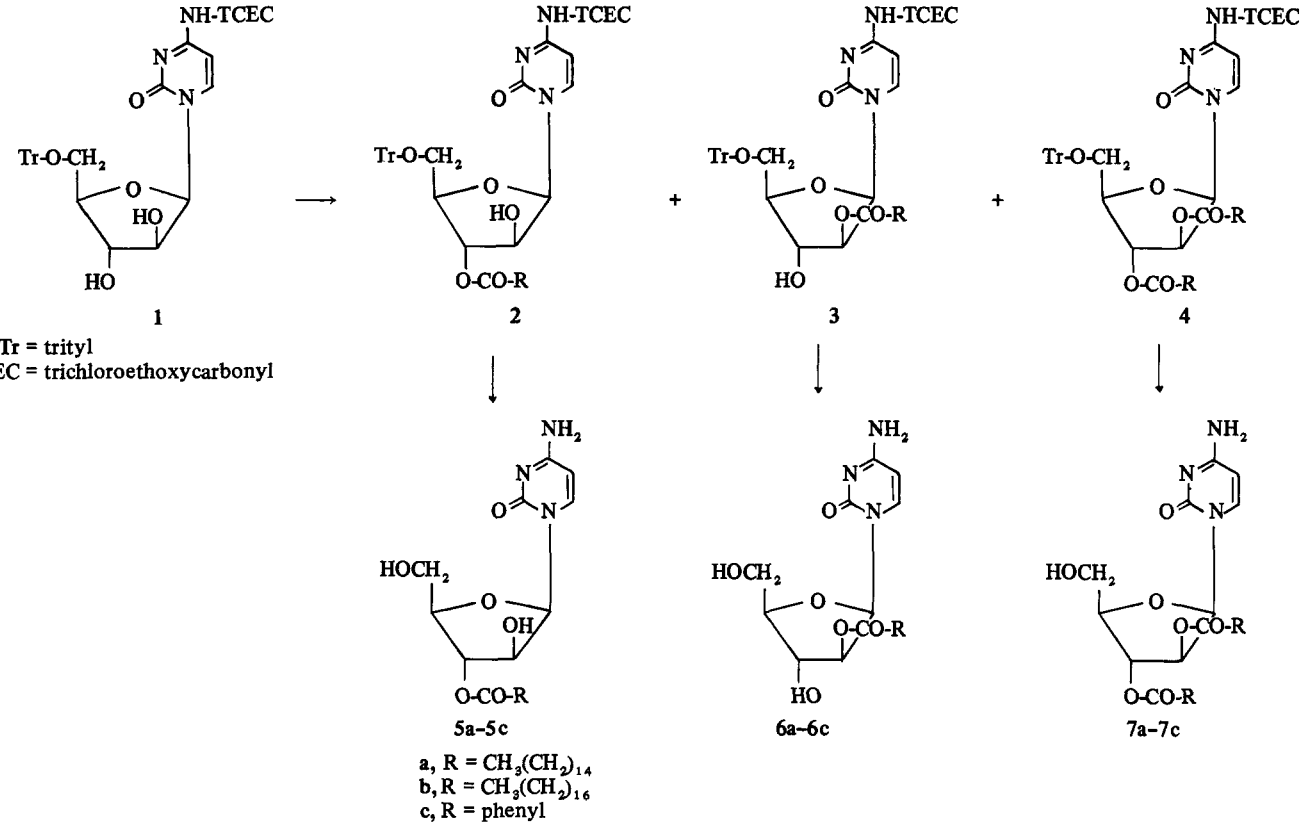

Table I

Ester of Ara-C	Acylating agent	Recryst solvent	Mp, °C (uncorrected capillary mp)	Analyses
2',3'-*O*-Dipalmitoyl (7a)	Palmitic anhydride	Me_2CO	144–145	C, H, N
3'-*O*-Palmitoyl (5a)	Palmitic anhydride	Me_2CO or MeOH	174–176	C,[a] H,[b] N[c]
2'-*O*-Palmitoyl (6a)	Palmitic anhydride	Me_2CO	136–138[d]	C, H, N
2',3'-*O*-Distearoyl (7b)	Stearoyl chloride	Me_2CO	143–145	C, H, N
3'-*O*-Stearoyl (5b)	Stearoyl chloride	Me_2CO or MeOH	175.5–177	C, H, N
2'-*O*-Stearoyl (6b)	Stearoyl chloride	MeOH–H_2O	140–145[e]	C, H, N[f]
2',3'-*O*-Dibenzoyl (7c)	Benzoyl chloride	$CHCl_3$	133–135[g]	C, H, N
3'-*O*-Benzoyl (5c)	Benzoyl chloride	Me_2CO	208–210[h]	C, H, N
2'-*O*-Benzoyl (6c)	Benzoyl chloride	MeOH	212–214[i]	C, H, N

[a] C: calcd, 62.34; found, 61.79. [b] H: calcd, 9.00; found, 8.34. [c] N: calcd, 8.72; found, 8.12. [d] Product obtained as monohydrate. [e] Obtained as monohydrate, sinters at 112°. [f] N: calcd, 7.96; found 8.52. [g] Hemihydrate, sinters at 128°. [h] Slight decomp. [i] Mmp with 3'-*O*- was 182–191°.

Table II. Nmr Data for 2'-*O*- and 3'-*O*-Esters of 1-β-D-Arabinofuranosylcytosine (Ara-Cytidine)

Compd	δ, ppm[a] H-1'	H-2'	H-6	H-5
3'-*O*-Stearoyl of ara-cytidine (5a)	6.0 d	4.98 m	7.62 d	5.72 d
2'-*O*-Stearoyl of ara-cytidine (6a)	6.20 d	5.20 t	7.65 d	5.70 m
2'-*O*-Benzoyl of ara-cytidine (6c)	6.32 d	5.50 t		

[a] d = doublet, m = multiplet, t = triplet.

ara-C. The possibility that this material contained a modified trichloroethoxycarbonyl group seems plausible but was not further investigated.§

The 2',3'-diester derivatives of 5'-*O*-trityl-N^4-trichloroethoxycarbonyl-ara-C (*ca.* 12 g) were stirred with 200 ml of 80% AcOH–20% H_2O and 12 g of Zn dust for 16 hr at room temperature. This effectively removed all of the trichloroethoxycarbonyl group; but after filtering the Zn, it was sometimes necessary to warm the aqueous AcOH filtrate until a small residual quantity of the 5'-*O*-trityl group was also hydrolyzed. After removal of the AcOH solvent *in vacuo*, the residue was dissolved in $CHCl_3$, washed with H_2O, and concd to a solid. The final product was recrystallized from Me_2CO. Sometimes a preliminary clean-up of the crude 2,3-diester-ara-C on a silica gel column using a solvent such as $CHCl_3$–MeOH (95:5) for elution gave a better product for the final crystallization.

The general procedure for obtaining 3'-*O*- and 2'-*O*-monoesters from their protected intermediates was similar to that used for preparing the 2'-*O*,3'-*O*-diesters in the previous paragraph. After removal of the 5'-*O*-trityl and N^4-trichloroethoxycarbonyl pro-

§ When trichloroethoxycarbonyl groups are removed by mild treatments such as zinc acetate–MeOH, an intermediate product can be identified on tlc plates which has a lower R_f than the parent compound and disappears when hydrolysis is complete (D. T. Warner, unpublished data).

Table III. Antileukemic Activity (L1210 Leukemic Mice) of Ara-Cytidine Esters[a]

Compd	*ara*-C derivative	Median Survival (days)	%ILS[b]
	Control	9.0	
7b	2',3'-Distearate	8.5	0
7a	2',3'-Dipalmitate	10.5	17
7c	2',3'-Dibenzoate	8.5	0
6b	2'-Stearate	12.5	39
6c	2'-Benzoate	10.0	11
5c	3'-Benzoate	13.0	45
5b	3'-Stearate		292[c]
	5'-Benzoate		185[c]
	5'-Stearate		216[c]
	5'-Palmitate		>300[d]

[a] Single dose treatment (200 mg/kg) ip, 1 day after tumor inoculation. [b] % increase in life-span calculated from median survivals. [c] Data from other experiments (G. L. Neil, unpublished data and ref 9). [d] 4/8 mice treated were cured (45-day survivors) at 100 mg/kg (ref 9).

tecting groups, the 2'-*O*- or 3'-*O*-monoesters were crystd from various solvents as indicated in Table I. The nmr data for the 2'-*O*- and 3'-*O*-esters are compiled in Table II.

Biological Testing of 2'- and 3'-Esters. Methods used in the testing of these compounds for antileukemic activity and immunosuppressive activity were similar to those used for 5'-esters of aracytidine.[9] Test data for the leukemic mice are indicated in Table III. All compounds were administered intraperitoneally (ip) as suspensions in 0.2 ml of aqueous methylcellulose to female BDG mice, one day after ip inoculation with 1×10^5 L1210 cells/mouse. The dose employed was 200 mg/kg. Groups of 8 mice were used. Per cent increase in life-span (% ILS) was calculated from median survivals of treated and control (untreated) groups. Data for some of the 5'-esters are included for comparison. The immunosuppressive activities of all of the compounds prepared in this work were consistently lower than the activities of the 5'-*O*-esters. Consequently no detailed results are included.

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Synthesis of Some Analogs of Angiotensin II as Specific Antagonists of the Parent Hormone†

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Syntheses of [8-cyclohexylalanine]-, [Val[8]]-, [Leu[8]]-, [Phe[4], Ala[8]]-, [Suc[1], Phe[4], Tyr[8]]-, and [Sar[1], Ile[8]]-angiotensin II was carried out by solid-phase technique to study factors responsible for agonistic or antagonistic properties in angiotensin II. While pressor response decreased, when position 8 (Phe) of angiotensin II was replaced with 3-amino-4-phenylbutyric acid (10%), DL-3-amino-2-benzylpropionic acid (1%), cyclohexylalanine (20%), valine (0.5%), or leucine (0.3%), antagonistic effect enhanced in the same order; analogs with valine and leucine were found to be very potent and specific antagonists of angiotensin II. The antagonistic properties of [Ile[8]]angiotensin II increased when aspartic acid in position 1 was substituted with sarcosine. However, antagonistic activity decreased when position 1 (Asp) in [Phe[4],Tyr[8]]angiotensin II was replaced with the succinic acid residue or when Tyr in position 4 of [Ala[8]]-angiotensin was replaced with Phe.

Structural modification of angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) indicates that replacement of the aromatic residue (Phe) by an aliphatic group in position 8 invokes antagonistic activity.[2-7] To further evaluate the potential of this modification, phenylalanine in position 8 was replaced by: (a) cyclohexylalanine (V), to delineate the contribution of aromaticity or ring size for myotropic response; (b) valine and leucine, to investigate the nature of aliphatic side chain required for potent antagonism; (c) 3-amino-4-phenylbutyric acid (III) or DL-3-amino-2-benzylpropionic acid (VI), to study the importance of the position of the aromatic side chain in relation to the remainder of the peptide chain for myotropic response. Transposition of the amino acid residues of positions 4 (Tyr) and 8 (Phe) in angiotensin II also invoked antagonistic activity to the parent hormone;[8] [Phe[4], Ala[8]] angiotensin II was synthesized to investigate the contribution of position 4 in relation to position 8 for the antagonistic effect. [Sar[1], Ile[8]]-, and [Suc[1], Phe[4], Tyr[8]]angiotensin II were synthesized to possibly improve the antagonistic properties.

Synthesis and Purification of Analogs. The solid-phase procedure of peptide synthesis[9] suffers from several shortcomings; the most serious of these is the formation of failure sequences and truncated sequences.[10,11] These are caused by incomplete deblocking or incomplete coupling. Also, mechanical shaking may break polymer beads, thereby exposing dormant sites, where coupling could take place to give rise to shortened chains. To avoid these difficulties, the Boc group was deblocked with CF_3COOH in CH_2Cl_2,[12,13] each coupling step was carried out twice,[14,15] and the poly-

†This investigation was partially supported by Grant HL-6835 from the National Heart and Lung Institute and partially by General Research Support Grant FR-5674. Presented in part at the 162nd National Meeting of the American Chemical Society, Washington, D. C., Sept 12-17, 1971.[1] Abbreviations used are: L-cyclohexylalanine = Cha; 3-amino-4-phenylbutyric acid = Apb; 3-amino-2-benzyl propionic acid = Apib. (In earlier publications[2,7] this compound was referred to as 3-amino-3'-phenylisobutyric acid.) Unless stated otherwise, all amino acids are of the L variety.