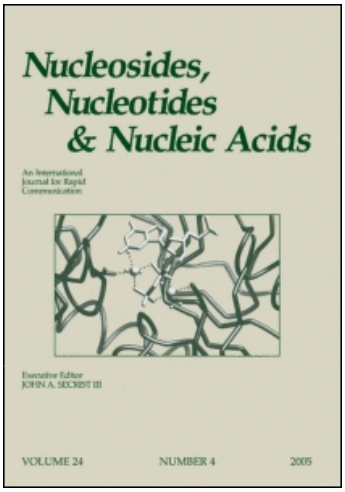

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Castro-pichel, Julia , García-López, Ma. Teresa , Herranz, Rosario and Pérez, Concepción(1990) '5'-*O*-[*N*-(Amnoacyl)Sulfamoyl]Nucleosides. Synthesis and Antiviral and Cytostatic Activities', Nucleosides, Nucleotides and Nucleic Acids, 9: 7, 985 — 1000
To link to this Article: DOI: 10.1080/07328319008045213
URL: http://dx.doi.org/10.1080/07328319008045213

NUCLEOSIDES & NUCLEOTIDES, 9(7), 985-1000 (1990)

5'-O-[N-(AMINOACYL)SULFAMOYL]NUCLEOSIDES. SYNTHESIS AND ANTIVIRAL AND CYTOSTATIC ACTIVITIES

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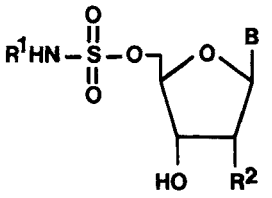
Abstract: 5'-O-[N-(Aminoacyl)sulfamoyl]-uridines and -thymidines **4a-12a** and **4b-12b** have been synthesized and tested against Herpes Simplex virus type 2 (HSV-2) and as cytostatics. Condensation of 2',3'-O-isopropylidene-5'-O-sulfamoyluridine and 3'-O-acetyl-5'-O-sulfamoylthymidine with the N-hydroxysuccinimide esters of Boc-L-Ser(Bzl), (2R,3S)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid [(2R,3S)-N-Z-AHPBA], (2R,3S)- and (2S,3R)-N-Boc-AHPBA gave **4a,b-7a,b**, which after removal of the protecting groups provided **10a,b-12a,b**. A study of the selective removal of the O-Bzl protecting group from the L-Ser derivatives **4a,b**, without hydrogenation of the pyrimidine ring, has been carried out. Only the fully protected uridine derivatives **4a-7a** did exhibit high anti-HSV-2 activity, and none of the synthesized compounds showed significant cytostatic activity against HeLa cells cultures.

Replacement of the 5'-phosphate group in nucleotides by the non-ionized sulfamate residue has been widely used in chemotherapy, with the aim of avoiding enzymatic cleavage or failure to cross the cell membrane.[1-3] Ascamycin (**1a**) is a naturally occurring 5'-O-[N-(L-alanyl)sulfamoyl]substituted nucleoside having selective antibacterial activity as compared to dealanylascamycin (**1b**).[5,6] Recently, we have synthesized and tested against Herpes Simplex virus type 2 (HSV-2) a series of 5'-O-[N-alkyl, -acyl and -aminoacyl)sulfamoyl] pyrimidine nucleosides (**2**), and we have found that the nature of the substituent attached to the sulfamoyl group affects the antiviral effect.[7,8] These

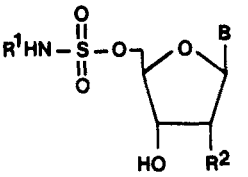


1a: R^1 = L-Ala ; R^2 = OH ; B = 2-chloroadenine

1b: R^1 = H ; R^2 = OH ; B = 2-chloroadenine

2: R^1 = Alkyl , acyl , L-Ala , D-Ala , L-Phe , Gly

R^2 = OH , H ; B = uracyl , cytosine , thymine



1a: R^1 = L-Ala ; R^2 = OH ; B = 2-chloroadenine

1b: R^1 = H ; R^2 = OH ; B = 2-chloroadenine

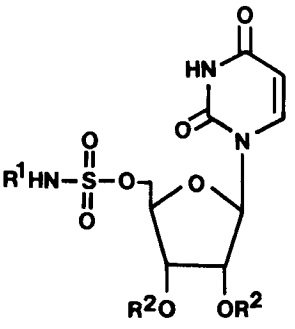
2: R^1 = Alkyl , acyl , L-Ala , D-Ala , L-Phe , Gly

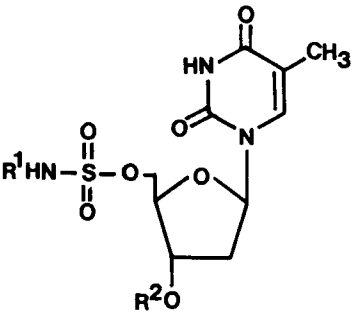
R^2 = OH , H ; B = uracyl , cytosine , thymine

facts prompted us to prepare new 5'-O-[N-(aminoacyl)sulfamoyl]substituted analogues in which the aminoacyl residues are amino hydroxy acids, such as L-serine and (2R,3S)- and (2S,3R)-3-amino-2-hydroxy-4-phenylbutanoic acid (AHPBA). This last amino hydroxy acid is essential for the biological properties of bestatin [(2S,3R)-AHPBA-L-Leu], a natural immunomodifier dipeptide with antitumor and antimicrobial activities.[8] The present paper describes the synthesis and anti-HSV-2 and cytostatic activities of these new 5'-O-[N-(aminoacyl)sulfamoyl] nucleoside derivatives.

CHEMISTRY

The 5'-O-[N-(aminoacyl)sulfamoyl]nucleosides **9a,b-11a,b** were obtained by aminoacylation of the appropriate protected 5'-O-sulfamoyl nucleoside **3a**[8] or **3b**,[10] followed by deprotection of the amino acid and nucleoside residues. Aminoacylations were carried out according to the method, free of racemization in the amino acid, employed in our laboratories for the synthesis of **1a**, which involves the reaction of the



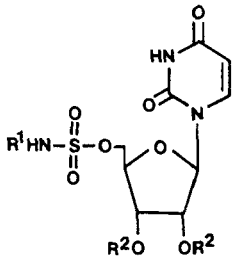


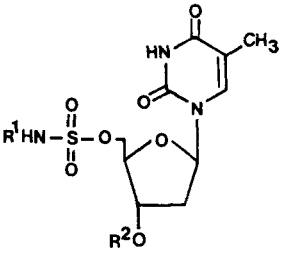
	R^1	R^2
3a	H	$C(CH_3)_2$
4a	Boc-*L*-Ser(Bzl)	$C(CH_3)_2$
5a	(2*R*,3*S*)-N-Z-AHPBA	$C(CH_3)_2$
6a	(2*R*,3*S*)-N-Boc-AHPBA	$C(CH_3)_2$
7a	(2*S*,3*R*)-N-Boc-AHPBA	$C(CH_3)_2$
8a	Boc-*L*-Ser	$C(CH_3)_2$
10a	*L*-Ser	H
11a	(2*R*,3*S*)-AHPBA	H
12a	(2*S*,3*R*)-AHPBA	H

	R^1	R^2
3b	H	Ac
4b	Boc-*L*-Ser(Bzl)	Ac
5b	(2*R*,3*S*)-N-Z-AHPBA	Ac
6b	(2*R*,3*S*)-N-Boc-AHPBA	Ac
7b	(2*S*,3*R*)-N-Boc-AHPBA	Ac
8b	Boc-*L*-Ser	AC
10b	*L*-Ser	H
11b	(2*R*,3*S*)-AHPBA	H
12b	(2*S*,3*R*)-AHPBA	H

Z = $C_6H_5CH_2OCO$; Boc = $(CH_3)_3COCO$

5'-sulfamoyl nucleosides with the N-hydroxysuccinimide esters of the protected amino acids in DMF, in the presence of DBU.[11] Following this method, we firstly prepared the fully protected 5'-O-[N-(aminoacyl)-sulfamoyl] nucleosides **4a,b** and **5a,b**, but all attempts to remove the Z group from **5a,b** were unsuccessful. Thus, hydrogenation using 10% Pd/C as catalyst led exclusively to the hydrogenation of the pyrimidine ring, while with formic acid and palladium black[12] there was not reaction. Finally, treatment with trimethylsilyl iodide (TMSI)[13] led to the breaking of the sulfamoyl-O-nucleoside bond and to the isolation of 5'-deoxy-5'-iodouridine. These results led us to synthesize the N-Boc protected analogues **6a,b** and **7a,b** from (2R,3S)- and (2S,3R)-N-Boc-AHPBA respectively. These N-Boc protected amino acids were prepared by catalytic hydrogenation of the corresponding N-Z-amino acid methyl esters,[14] in the presence of di-t-butyldicarbonate.[15] The resulting





	R^1	R^2
3a	H	$C(CH_3)_2$
4a	Boc-L-Ser(Bzl)	$C(CH_3)_2$
5a	(2R,3S)-N-Z-AHPBA	$C(CH_3)_2$
6a	(2R,3S)-N-Boc-AHPBA	$C(CH_3)_2$
7a	(2S,3R)-N-Boc-AHPBA	$C(CH_3)_2$
8a	Boc-L-Ser	$C(CH_3)_2$
10a	L-Ser	H
11a	(2R,3S)-AHPBA	H
12a	(2S,3R)-AHPBA	H

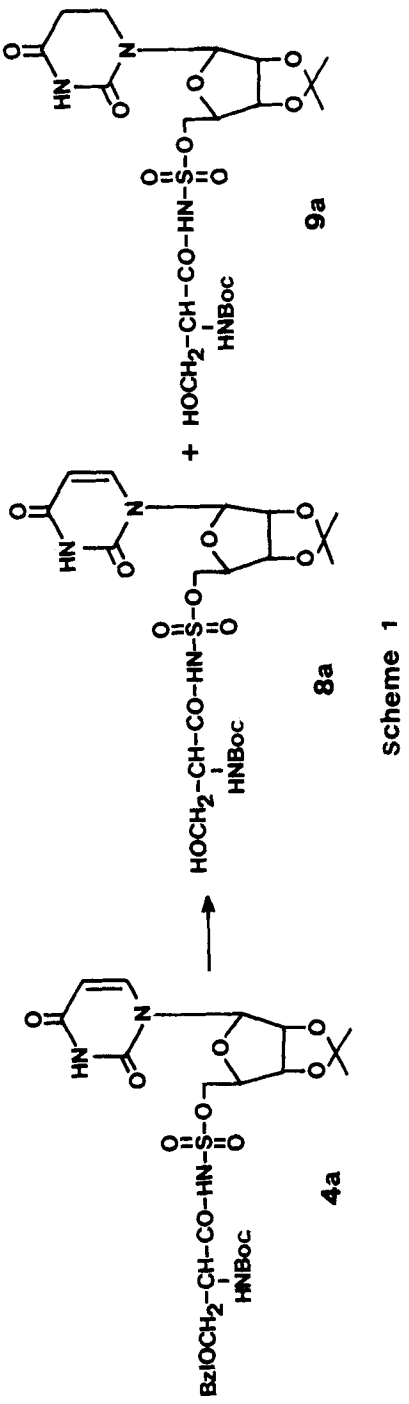
	R^1	R^2
3b	H	Ac
4b	Boc-L-Ser(Bzl)	Ac
5b	(2R,3S)-N-Z-AHPBA	Ac
6b	(2R,3S)-N-Boc-AHPBA	Ac
7b	(2S,3R)-N-Boc-AHPBA	Ac
8b	Boc-L-Ser	Ac
10b	L-Ser	H
11b	(2R,3S)-AHPBA	H
12b	(2S,3R)-AHPBA	H

$Z = C_6H_5CH_2OCO$; $Boc = (CH_3)_3COCO$

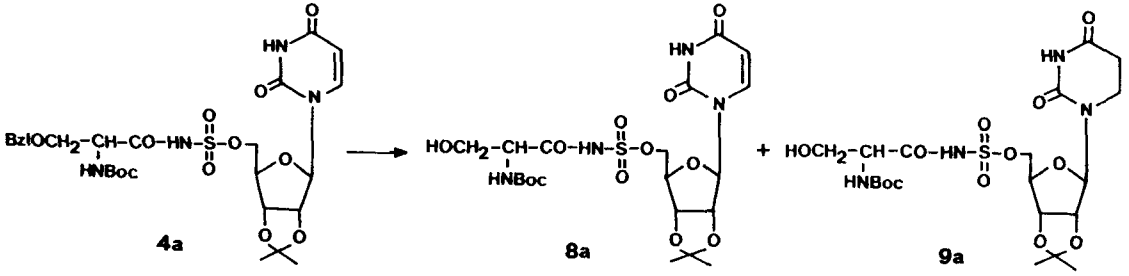
N-Boc amino acid methyl esters were saponified to the corresponding acids, which were used for the aminoacylation without further purification. Treatment of **6a,b** and **7a,b** with trifluoroacetic acid (TFA) and, in the case of **6b** and **7b**, subsequent deacetylation with methanolic ammonia, provided the desired fully deprotected analogues **10a,b** and **11a,b** respectively.

Difficulties were also found in several attempts to remove the O-benzyl protecting group from the serine derivative **4a**. Thus, catalytic hydrogenation in MeOH, using 10% Pd/C as catalyst (Scheme 1), led to a 3:2 mixture of the corresponding debenzylated compound **8a** along with the debenzylated 5,6-dihydro derivative **9a**, which could not be separated by chromatography. On the other hand, treatment of **4a** with TMSI in TFA, in the presence of thioanisol[16], led to the removal of the N-Boc and O-isopropylidene groups, but not of the benzyl group. Therefore, with the aim of removing selectively the benzyl group without hydrogenation of the pyrimidine ring, we carried out a study of the hydrogenation of **4a**, using different catalyst[17] and different conditions (Table 1). As indicated in this table, only the hydrogenation of **4a** with formic acid as hydrogen donor and using palladium black as catalyst[12] gave, selectively and with good yield, the debenzylated compound **8a** (entry 10). Similarly, the hydrogenation of the thymidine derivative **4b**, in the same conditions, with formic acid and palladium black yielded the corresponding debenzylated derivative **8b**. Removal of the Boc and isopropylidene groups, and Boc and acetyl protecting groups from the uridine and thymidine derivatives **8a** and **8b**, under the usual conditions, yielded **12a** and **12b**.

The aminoacylation site, in all these 5'-O-[N-(aminoacyl)sulfamoyl] nucleosides, was established in the protected compounds **4a,b-7a,b** according to their ^{1}H-NMR spectra (Table 2), which showed the absence of a signal at ≈ 7.6 ppm attributed to the SO_2NH_2 protons in the starting 5'-O-sulfamoylnucleosides **3a** and **3b**, and the presence of a singlet at ≈ 11.3 ppm corresponding to the 3-NH pyrimidine proton. The deprotected compounds **10a-12a** and **10b-12b** showed typical uridine and thymidine UV spectra (Table 3), confirming that aminoacylation in the pyrimidine ring had not taken place.



Scheme 1



Scheme 1

TABLE 1. Different conditions for the hydrogenation[a] of **4a**

Entry	Hydrog. Agent	Catalyst	H_2 Pressure (Psi)	Time (h)	**8a:9a** Ratio[b]
1	H_2	10% Pd/C	20	4	3:2
2	H_2	10% Pd/C	30	10	0:10
3	H_2	10% Pd/C	10	6	3:1
4	H_2	10% Pd/$BaSO_4$	2	10	5:1
5	H_2	5% Pd/$BaSO_4$	25	120	6:1
6	H_2	5% Pd/$BaSO_4$	40	36	6:1
7	H_2	Pd/$CaCO_3$	20	3	degradation compds.
8	H_2	Pd/Al_2O_3	20	4	6:1
9	NH_4HCO_2	10% Pd/C	-	30	no reaction
10	HCO_2	Pd black	-	10	10:0

[a] All the hydrogenations were carried out in MeOH at room temp., except for entry 9, which was carried out in refluxing MeOH.
[b] These ratios were determined by the ^{1}H-NMR spectrum of the mixture.

TABLE 2. Analytical and spectroscopic data of the protected 5'-O-[N-(aminoacyl)sulfamoyl]nucleosides

Comp.	Yield %	$[\alpha]_D^{25}$ [a]	Formula[b]	Relevant ^{1}H-NMR (δ) data[c]					
				Nucleoside				Aminoacyl	
				H-1'	$J_{1',2'}$ (Hz)	H-5'	NH-3	CH-α	CH-ß
4a	92	-58.4°	$C_{27}H_{36}N_4O_{12}S$	5.85	2.7	4.03	11.40	3.95	3.66, 3.59
5a	85	-25.8°	$C_{30}H_{34}N_4O_{12}S$	5.84	2.7	4.01	11.36	3.60	3.97
6a	78	-22.3°	$C_{27}H_{36}N_4O_{12}S$	5.84	2.6	4.02	11.36	3.56	3.90
7a	83	+19.4°	$C_{27}H_{36}N_4O_{12}S$	5.85	2.7	4.02	11.32	3.57	3.90
8a	94	-	$C_{20}H_{30}N_4O_{12}S$	5.85	2.9	4.02	11.35	3.72	3.54
4b	87	+ 2.4°	$C_{27}H_{36}N_4O_{12}S$	6.21	7.2	4.03	11.30	3.86	3.62
5b	86	-	$C_{30}H_{34}N_4O_{12}S$	6.20	5.6, 9.3	4.01	11.30	3.60	4.01
6b	72	- 8.8°	$C_{27}H_{36}N_4O_{12}S$	6.22	5.6, 5.4	4.03	11.25	3.57	3.91
7b	75	+24.3°	$C_{27}H_{36}N_4O_{12}S$	6.22	6.0	4.04	11.28	3.57	3.86
8b	92	-	$C_{20}H_{30}N_4O_{12}S$	6.22	5.8	4.04	11.30	3.72	3.54

[a] C=0.5 in MeOH. [b] Analytical results were within ± 0.4%. [c] DMSO-d_6 as solvent.

TABLE 3. Analytical and spectroscopic data of the deprotected 5'-O-[N-(aminoacyl)sulfamoyl]nucleosides

Comp.	Yield %	m.p.[a] (°C)	$[\alpha]_D^{25}$[b]	λ_{max}(nm) (log ε)	Formula[c]	Relevant ^{1}H-NMR (δ) data[d]				
						Nucleoside			Aminoacyl	
						H-1'	$J_{1',2'}$	H-5'	CH-α	CH-ß
10a	78	144-147	+ 2.6°	258 (3.836)	$C_{12}H_{18}N_4O_{10}S$	5.80	6.0	4.02	3.71	3.35 3.48
11a	85	167-170	+ 2.2°	258 (3.894)	$C_{19}H_{24}N_4O_{10}S$	5.76	3.9	4.31	3.92	3.50
12a	74	161-163	+21.9°	258 (3.908)	$C_{19}H_{24}N_4O_{10}S$	5.75	4.0	4.30	3.92	3.58
10b	80	165(dec)	+ 6.4°	264 (3.975)	$C_{13}H_{20}N_4O_9S$	6.23	7.5	4.05	3.39	3.54 3.76
11b	62	165-167	+ 6.8	264 (3.843)	$C_{20}H_{26}N_4O_9S$	6.11	7.0	4.30	3.86	3.62
12b	65	179(dec)	+15.1°	264 (3.806)	$C_{20}H_{26}N_4O_9S$	6.12	7.2	4.28	3.89	3.47

[a] From $CHCl_3$/MeOH. [b] C=0.5 in MeOH. [c] Analytical results were within ± 0.4%. [d] D_2O as solvent.

BIOLOGICAL RESULTS AND DISCUSSION

Antiviral Activity. All the compounds here reported were tested as antivirals against HSV-2 in Vero cells. None of the thymidine derivatives **4b-12b** showed antiviral activity in this assay (data not shown). The activities of the 5'-O-[N-(aminoacyl)sulfamoyl]uridine derivatives **4a-12a** are included in Table 4. For comparative purposes, 5'-O-[N-(L-Ala)sulfamoyl]uridine (**2**, R^1=L-Ala, R^2=OH, B=uracyl)[8] and acyclovir, a well known anti-HSV-2 agent, have been also tested under the same experimental conditions. As shown in the table, the fully protected compounds **4a-7a** exhibited high activity, specially marked in **4a** and **5a**, having benzyl and benzyloxycarbonyl portecting groups. However, the toxicity of these two compounds was also higher than that of the other active compounds. The deprotected analogues were inactive, with the exception of the (2R,3S)-AHPBA derivative **11a**, which showed a modest activity. As previously suggested for other related 5'-O-[N-(substituted)sulfamoyl]uridines, the influence of the protecting groups on the antiviral effect, could be due to differences in the transport of the compounds into the cell.[7,18]

Cytostatic Activity. None of the synthesized compounds showed significant cytostatic activity against HeLa cells. All of them were inactive at a concentration of 100 μg/mL, except for the protected uridine derivatives **4a** and **5a**, which had an ID_{50} of 10 and 12.5 μg/mL, respectively.

EXPERIMENTAL SECTION

CHEMICAL METHODS

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. The elemental analyses were determined on a Heraeus CHN-O-RAPID instrument. Optical rorations were determined on a Perkin-Elmer 141 polarimeter. UV spectra were recorded with a Perkin-Elmer 550 spectrometer. ^{1}H-NMR spectra were recorded with a Varian XL-300 (300 MHz) and a Varian EM-390 (90 MHz) spectrometers, using Me_4Si as internal standard. Analytical TLC was performed on a

TABLE 4. In vitro anti-HSV-2 activity and toxicity of the 5'-O-[N-(aminoacyl)sulfamoyl]uridine derivatives prepared

Compounds	CD_{50}[a] (μg/mL)	ED_{50}[b] (μg/mL) (TI[c])
2[d]	300	150 (2)
4a	20	2.5 (8)
5a	20	2 (10)
6a	200	25 (8)
7a	100	25 (4)
8a	>400	>400
10a	>400	>400
11a	400	200 (2)
12a	>400	>400
Acyclovir	300	0.5 (600)

[a] 50% cytotoxic dose, required to reduce the viability of normal uninfected cells by 50%.
[b] 50% Effective dose, to protect 50% of cells from the cytopathic effect of HSV-2.
[c] $TI=ED_{50}/CD_{50}$.
[d] **2**, R^1=L-Ala, B=uracyl, R^2=OH.

aluminium sheets coated with a 0.2 mm layer of silica gel 60 F_{254}, Merck. Column chromatography was performed on silica gel 60 (230-400 mesh), Merck. On TLC, the compounds were detected by UV light and by spraying with ninhydrine solution in EtOH or with 30% H_2SO_4 in EtOH.

General Procedure for the Conversion of N-Benzyloxycarbonyl-AHPBA derivatives into N-t-Butyloxycarbonyl-AHPBA Derivaties.

Di-t-butyl dicarbonate (545 mg; 2.5 mmol) and 10% Pd/C (250 mg) were added to a solution of (2R,3S)- or (2S,3R)-N-Z-APBHA methyl ester (750 mg; 2.3 mmol) in MeOH (60 mL) and the mixture was hydrogenated at room temperature and 15 psi of H_2 presure for 6 h. Then, the catalyst was filtered off and the solution evaporated to dryness to give the corresponding N-Boc methyl ester as a foam (675 mg; 95%).

(2R,3S)-3-t-Butyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid methyl ester. From (2R,3S)-N-Z-AHPBA methyl ester.[8] $[\alpha]^{20}_{578}$=+36° (c= 0.90, MeOH); ^{1}H-NMR ($CDCl_3$): δ = 1.38 (s, 9H, Boc), 2.88 (d, 2H, J=8.4 Hz, $\underline{CH_2}Ph$), 3.72 (s, 3H, OCH_3), 4.07 (d, 1H, J=2.2 Hz, C_2-H), 4.24 (m, 1H, C_3-H), 4.88 (d, 1H, J=9.5 Hz, NH), 7.53 (s, 5H, Ph).

Anal. Calcd. for $C_{16}H_{23}NO_5$: C, 62.12; H, 7.49; N, 4.53. Found: C, 61.98; H, 7.60; N, 4.71.

(2S,3R)-3-t-Butyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid methyl ester. From (2S,3R)-N-Z-AHPBA methyl ester.[8] $[\alpha]^{20}_{578}$=-36° (c=0.95, MeOH); ^{1}H-NMR ($CDCl_3$) the same than for its enantiomer (2R,3S)-N-Boc-AHBPA methyl ester.

Anal. Calcd. for $C_{16}H_{23}NO_5$: C, 62.12; H, 7.49; N, 4.53. Found: C, 62.23; H, 7.50; N, 4.62.

Saponification of N-Boc-AHPBA Methyl Esters. Synthesis of (2R,3S)- and (2S,3S)-N-Boc-AHPBA. NaOH (2.4 mmol) was added to a solution of the corresponding N-Z-AHPBA methyl ester (2 mmol) in a 1:1 dioxane: H_2O mixture (50 mL) and the mixture was stirred at room temperature for 1 h. Then, the reaction mixture was concentrated (≈ 20 mL), diluted with water (40 mL) and extracted with CH_2Cl_2 (3 x 40 mL). The aqueous phase was acidified to pH 3-4 with Dowex 50W-X4 resin. The resin was filtered and washed with CH_2Cl_2 (50 mL), and the aqueous phase was extracted with CH_2Cl_2 (3 x 50 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated to give quantitatively the corresponding N-Boc-AHPBA, which were used for the following step without further purification.

Aminoacylation of 5'-O-Sulfamoylnucleosides with Boc-L-Ser(Bzl), (2R,3S)-N-Z-AHPBA and (2R,3S)- and (2S,3R)-N-Boc-AHPBA (General Procedure for 4a,b-7a,b). Dicyclohexylcarbodiimide (1.36 g; 6 mmol) was added to a solution of the corresponding N-protected amino acid. (5 mmol) and N-hydroxysuccinimide (0.57 g; 5 mmol) in dry 1,2-dimethoxyethane (15 mL) at 0°C. The solution was stirred at 0-5°C for 20 h and, then, the dicyclohexylurea formed was filtered off and the filtrate evaporated to dryness. The residue was disolved in dry DMF (5

mL) and added to a solution of 2',3'-O-isopropylidene-5'-O-sulfamoyluridine[8] (**3a**) or 3'-O-acetyl-5'-O-sulfamoylthymidine[10] (**3b**) (5 mmol) and DBU (5 mmol) in dry DMF (20 mL), and the solution was stirred at room temperature for 2 h. Then, the reaction mixture was evaporated to dryness under reduced presure and the residue was purified by flash chromatography using $CHCl_3$/MeOH mixtures as eluents. The protected compounds thus obtained **4a,b, 5a,b, 6a,b** and **7a,b**, as foams, are summarized in Table 2.

Removal of the Benzyl Protecting Group in the 5'-O-[N-[Boc-Ser(Bzl)]sulfamoyl]nucleoside Derivatives. (General Procedure for 8a and 8b). A suspension of palladium black (250 mg) in 4.4 % formic acid in MeOH (15 mL) was added to a solution of **4a** or **4b** (1 mmol) in 4.4 % formic acid in MeOH (15 mL). The reaction mixture was stirred, under argon, at room temperture for 12 h. Then, the catalyst was filtered off and the filtrate evaporated to dryness. The residue was purified by flash chromatography, using $CHCl_3$/MeOH mixtures as eluents. The compounds **8a** and **8b**, thus obtained, are summarized in Table 2.

Removal of the Boc and Isopropylidene Protecting Groups (General Procedure for 10a, 11a and 12a). A solution of the corresponding protected compound **8a, 6a** or **7a** (0.5 mmol) in a 5:2 TFA:H_2O mixture (7 mL) was stirred at room temperature for 2 h. Then, the reaction mixture was evaporated to dryness at room temperature, and the residue was purified by flash chromatography, using $CHCl_3$/MeOH as eluent. Compounds **10a-12a** were crystalized from the proper solvent and their analytical and spectral data are summarized in Table 3.

Removal of the Boc and Acetyl Protecting Groups (General Procedure for 10b, 11b and 12b). A solution of the corresponding protected compound **8b, 6b** or **7b** (0.6 mmol) in a 5:2 TFA:H_2O mixture (10 mL) was stirred at room temperature for 2 h. Then, the reaction mixture was evaporated to dryness, the residue was disolved in a saturated ammonia solution in MeOH, and the solution was stirred at room temperature for 6 h. Then, the reaction mixture was evaporated and the residue crystalized from $CHCl_3$/MeOH. The compounds **10b, 11b** and **12b** thus obtained, are indicated in Table 3.

BIOLOGICAL MATERIALS AND METHODS

Antiviral Activity

Cells and cell cultures. Vero cells were grown in Dulbecco's modified Eagle medium with glutamine, supplemented with 10 % calf serum, and either 0.85 % $NaHCO_3$ for flask culture or 3.7 % for cultures in 24- and 96-well plates (Costar, Cambridge, Ma. USA), incubated in a 5 % CO_2 atmosphere, 95 % humidity and 37°C. The maintenance medium was supplemented with 2 % calf serum. Vero cells, media and sera were supplied by Flow Labs. Scotland, UK.

Viruses. Herpes Simplex virus type (HSV-2) Lovelace strain was obtained from the Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Spain, by courtesy of Dr. R. Nájera.

In vitro antiviral assay. Confluent Vero cell monolayers growing in 24-well plates were virus infected with a MOI (multiplicity of infection) of 0.5 pfu plaque forming unit per cell. After 90 min of adsorption the virus was removed and the cells were further incubated in maintenance medium containing various concentrations of the test compounds. Virus infected cultures without compound and uninfected cells treated with the different compounds were included as controls. After 48 h incubation at 37°C, the cell monolayer was examined under a phase-contrast microscope and the cytopathic effect was recorded. Toxicity was also estimated under the microscope in uninfected cells.

Cytostatic Activity. The previously described method[19] was followed. Minimal Eagle's medium (Difco, code 5675) supplemented with 10% fetal calf serum (Difco) was used. HeLa cells (10^5 cells/mL) were incubated at 37°C in Leighton tubes. After 2-3 h, the cells were attached to the glass, and the compound to be tested, suspended in sterile saline containing 0.05% (v/v) Tween 80, was then added. The volume of this suspension was 10% of the final incubation mixture. Incubation was carried out at 37°C for 72 h. As a positive control, 6-mercaptopurine was always included ($ED_{50} \approx 0.1$ μg/mL). Cell growth was estimated by measuring the cell proteins following the colorimetric method of Oyama and Eagle.[20]

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Received May 3, 1990.