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Synthesis and Physico-Chemical Properties of 6- and 7-Monosubstituted Pyrazino|2,3-c|-1,2,6-thiadiazine 2,2-Dioxides

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Summary

Selective procedures for the synthesis of 6- and of 7-monosubstituted derivatives of pyrazino|2,3-c|-1,2,6-thiadiazine 2,2-dioxide and their reactions with N-bromosuccinimide are reported. The structures of the compounds are discussed on the basis of ^{1}H-, ^{13}C-NMR and UV spectroscopic data. The pK_a values of the compounds have also been determined.

Introduction

Pyrazino|2,3-c|-1,2,6-thiadiazine 2,2-dioxides are a group of heterocycles which can be regarded as SO_2 analogs of pteridines. Their synthesis usually involves condensation of an appropriate o-diaminothiadiazine with a carbonyl compound and thus, 6,7-disubstituted and 7-oxo derivatives have been obtained from symmetrical 1,2-dicarbonyl compounds (1) and α-ketoacid esters (2) respectively. As in the related pteridine series, problems regarding regioselectivity arise when using unsymmetrical 1,2-dicarbonyl compounds (3) and thus, mixtures of the 6- and 7-substituted isomers are to be expected.

In this paper, we report selective procedures for the synthesis of 6- and of 7-monosubstituted derivatives of pyrazino|2,3-c|thiadiazines and their reaction with N-bromosuccinimide (NBS) to give the corresponding bromo compounds, versatile intermediates for further structural modifications by nucleophile displacement.

Results and Discussion

The first reaction studied was that between 3,4,5-triamino-2H-1,2,6-thiadiazine 1,1-dioxide (**1**) (4) and α-aldehydoketones. In principle, the more reactive aldehydo carbonyl group would react with the 4-amino group and afford 7-substituted derivatives. However, when **1** was treated with phenylglyoxal in water/hydrochloric acid a mixture of the 6-phenyl and 7-phenyl derivatives (**2** and **3**) was isolated. Even in the presence of sodium bisulfite, which might be expected to complex preferentially with the aldehydo group and thereby, afford the 6-phenyl isomer [as it does in some pteridine synthesis (3)] a mixture was obtained.

When the reaction between the triaminothiadiazine **1** and phenylglyoxal was carried out in acetic acid only the 7-phenyl derivative **3** was formed. Under none of the conditions studied was it possible to obtain preferentially the 6-phenyl derivative **2** from phenylglyoxal; nevertheless, this compound was selectively obtained in the reaction of **1** with ω-hydroxyiminoacetophenone, based on the two-step condensation of an α-ketocarboxaldoxime with a 5,6-diaminopyrimidine (5).

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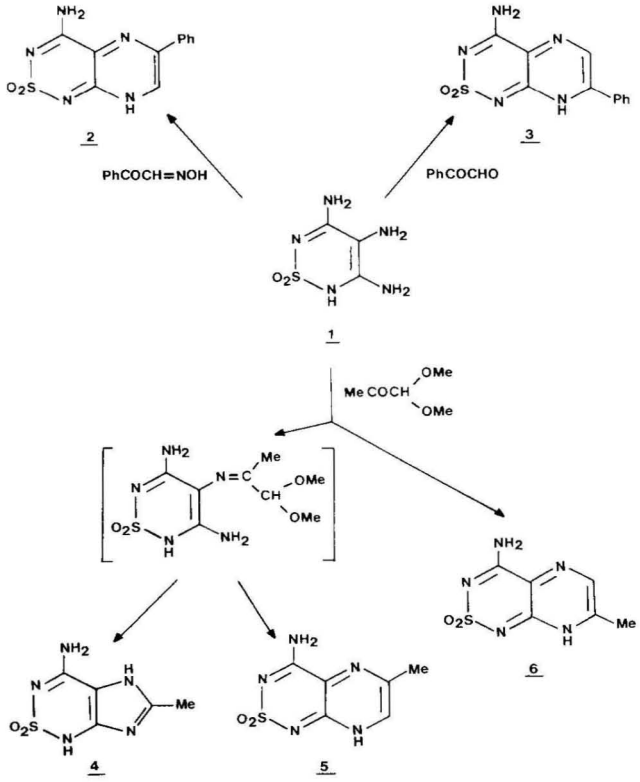
On the other hand, condensation of **1** with methylglyoxal dimethylacetal in water and hydrochloric acid afforded mixtures of the corresponding 6- and 7-methyl derivatives **5** and **6**, together with 4-amino-6-methyl-1H,5H-imidazo[4,5-c]-1,2,6-thiadiazine 2,2-dioxide (**4**). This fused five-membered ring, identical in all respects to the compound obtained in the reaction between **1** and acetaldehyde (6), is the only product obtained when the condensation is performed in acetic acid. Since triaminothiadiazine **1** and acetic acid do not yield compound **4**, a plausible mechanism for its formation must involve nucleophilic addition of the 4-amino group to give the Schiff base of methylglyoxal dimethylacetal followed by hydrolysis and oxidative cleavage (see Scheme 1).

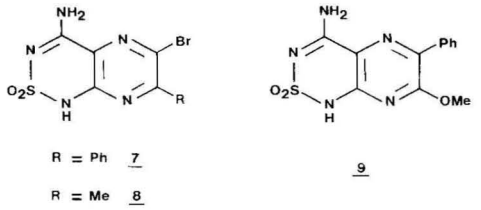


The 7-methyl derivative **6** could selectively be obtained by condensation of triaminothiadiazine **1** with methylglyoxal dimethylacetal under argon atmosphere in acetic acid and a few drops of hydrochloric acid in order to cleave the acetal group. In these conditions, the free aldehyde attacks the more reactive 4-amino group affording exclusively the 7-methyl derivative **6**.

Reaction of **1** with pyruvaldehyde 1-oxime afforded a mixture of the 6- and 7-methyl derivatives **5** and **6** which after crystallization yielded pure 6-methyl compound **5**. However, a more selective procedure to obtain **5** is to react **1** with methylglyoxal dimethylacetal in acetic acid under an argon atmosphere, in order to avoid oxidation to the purine analog **4**. Subsequent addition of hydrochloric acid cleaves the acetal grouping and thus affords pure **5** (Scheme 1).

Treatment of the 7-phenyl and 7-methyl pyrazinothiadiazines **3** and **6** with NBS in methanol afforded the corresponding 6-bromo derivatives **7** and **8** in acceptable yields. This method to introduce a bromine atom in the 6-position is not known in the related pteridine series.

This procedure did not work so well with the 6-substituted compounds and under more drastic conditions complex mixtures together with starting material were obtained. However, in the case of the 6-phenyl derivative, the 7-methoxy compound **9** could be isolated indicating that bromination must have taken place.



The structures of the newly synthesized compounds have been established based upon analytical and spectroscopic data. The ^{13}C-NMR chemical shifts are gathered in Table 1. The C-6 signals of the 7-substituted compounds and the C-7 signals of the 6-substituted ones were assigned unequivocally (DEPT 135°, coupled spectra) and the rest of the signals by comparison with the data of related compounds (7). With the chemical shifts of the carbons of the pyrazine moiety it was possible to assign structures to the 6- and 7-substituted compounds since C-6 is shielded in relation to C-7. In agreement with this fact, the signals of the protons belonging to C-7 appear in the ^{1}H-NMR spectra at lower field (**2**, $\delta = 9.27$; **5**, $\delta = 8.56$) than those corresponding to the 6- position (**3**, $\delta = 8.96$; **6**, $\delta = 8.33$).

More information about the predominant molecular species in solution can be gained from the UV spectra which are based on the spectrophotometrically determined pK_a values (8) (Table 2). The position of the tautomeric equilibrium can be depicted from the two UV long wavelength absorption bands of the neutral species since the longest band at 400 nm and higher is characteristic for the H-8 tautomer whereas the second band between 340 and 370 nm indicates the presence of the H-1 isomer. This assignment has already been proved by former investigations (1). It is interesting to note that the tautomeric equilibrium

Table 1. ^{13}C NMR Chemical shifts (ppm) of pyrazino[2,3-c]-1,2,6-thiadiazine 2,2-dioxides (DMSO-d_6)

Compound	C-4	C-7	C-8a	C-6	C-4a	R			
2[a]	158.5	145.9	147.8	144.7	121.1	134.6	129.4	128.7	126.4
3[b]	158.4	155.0	148.7	135.1	121.0	134.5	131.3	129.1	127.6
5[a]	158.8	148.7	149.5	144.0	120.3	20.2			
6[a]	159.1	158.6	148.4	137.5	119.3	21.3			
7[a]	158.3	157.3	147.6	128.6	120.8	136.2	130.1	129.3	128.0
8	159.9	157.5	147.7	130.8	119.9	24.0			
9	159.9	158.6	147.4	135.9	113.8	134.4	129.0	128.1	55.0

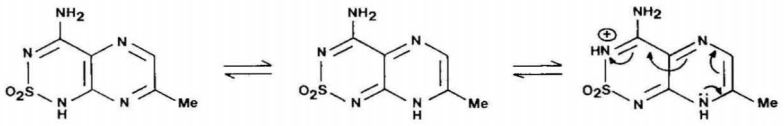
[a] DEPT 135° were recorded. [b] Coupled spectra.

Table 2. Physical data of pyrazino[2,3-c]-1,2,6-thiadiazine 2,2-dioxides

Compound	pK_a in H_2O	UV-absorption spectra $\lambda_{max\ (nm)}$				log ε				pH	Molecular form
4-Amino-6-methyl-8H- (**5**)	−2.29	260	(308)	386	410	4.10	(3.17)	3.66	3.69	−4.3	+
	3.56	248	(268)	343	413	4.01	(3.76)	3.71	3.25	1.0	o
		260	(296)	388		4.17	(3.10)	3.73		6.0	−
4-Amino-7-methyl-8H- (**6**)	−2.25	259	(320)		398	4.07	(3.06)		3.87	−4.3	+
	3.79	258		336	402	3.98		3.64	3.70	1.0	o
		260	(320)	378		4.13	(3.08)	3.80		6.0	−
4-Amino-6-phenyl-8H- (**2**)	−2.76	237	292		438	3.93	4.37		3.77	−4.3	+
	3.40	(218)	278	364	437	(4.14)	4.37	3.75	3.40	0.0	o
		216	286		404	4.12	4.31		3.83	6.0	−
4-Amino-7-phenyl-8H- (**3**)	−2.87	(217)	271	313	423	(4.13)	3.98	3.80	4.21	−4.9	+
	3.60	(224)	265	358	426	(4.11)	4.00	4.21	3.29	1.0	o
		(230)	270	(298)	397	(4.13)	4.26	(3.99)	4.01	6.0	−
4-Amino-6-bromo-7-methyl-1H- (**8**)	−3.63	215	270		424	4.09	4.15		3.89	−5.5	+
	3.01	202	255	348	421	4.21	4.14	3.87	2.91	1.0	o
		208	266		391	4.25	4.25		3.80	5.0	−
4-Amino-6-bromo-7-phenyl-1H- (**7**)	−4.29	216	277	341	444	4.21	4.13	3.50	4.09	−6.2	−
	2.83	(228)	260	364	439	(4.08)	4.15	4.10	2.66	0.0	o
		203	272	(308)	405	4.43	4.32	(3.61)	3.92	6.0	−
4-Amino-7-methoxy-6-phenyl-1H- (**9**)	−3.34	229	(266)	277	346	4.24	(4.14)	4.15	4.16	1.0	o
		237	(276)	293	375	4.14	(4.20)	4.25	4.10	6.0	−

( ) = Shoulder; + = monocation; − = monoanion; o = neutral form.

depends to a large extent on the substitution in the 6- and 7-position since a comparison of the extinction coefficients of **5** and **6**, for example, indicates a higher percentage of the H-1 over the H-8 tautomer in the former compound and an almost equal amount of the two tautomers in the latter isomer. Cation formation takes place only in very strong acidic medium since all these compounds own very weak basic properties as seen from the low basic pK_a values. The preferred protonation site seems to be N-3 forming a long mesomeric cation species with the H-N (8) group as the electron-donating center. Shift of the tautomeric equilibrium towards this molecular form can be depicted again from the long wavelength absorption bands and their extinction coefficients. Compound **6** obviously exists almost predominantly in one cation form whereas its 6-methyl isomer **5** consists of a 1 : 1 cation mixture.



In the 6-phenyl- (**2**) and 7-phenyl- (**3**) series similar considerations are valid. Here, however, the presence of the phenyl substituent at C-7 shifts the equilibrium even more strongly to the H-1 site from steric reasons. Protonation, on the other hand, causes again a pronounced shift of the equilibrium towards the H-8 cation species. Introduction of a bromine atom into the 6-position of **3** and **6** induces further changes by repressing the H-8 tautomers to a much greater extent. Finally, the UV spectrum of **9** clearly supports the H-1 tautomeric molecular form.

Acknowledgement

We thank Mrs. M. Bischler for the determinations of the pK_a values and the measurements of the UV spectra. Financial support, which is gratefully appreciated, was provided by MEC and CICYT (Spain).

Experimental Part

Melting points are uncorrected. The UV spectra were recorded with a Perkin-Elmer Lambda 5 instrument. The ^{1}H- and ^{13}C-NMR spectra were recorded with a Varian 390 and a Bruker WP-SY-80 spectrometer (TMS as internal standard). Mass spectra were obtained at 70 eV with a VG-12-250 spectrometer.

4-Amino-6-phenyl-8H-pyrazino[2,3-c]-1,2,6-thiadiazine 2,2-dioxide (**2**)

A suspension of **1** (1 g, 5.65 mmol) and ω-hydroxyiminoacetophenone (1.4 g, 9.46 mmol) in water (35 ml) and methanol (20 ml) with concentrated HCl (0.5 ml) was stirred at room temperature for 24 h. After removal "in vacuo" of the methanol, the precipitate was filtered, washed with chloroform and recrystallized from water : methanol to give 1.28 g (84%), mp. 302–304 °C. ^{1}H-NMR (90 MHz, DMSO-d_6): δ = 9.27 (s, 1H, H-7), 8.70 (br. s, 2H, NH_2), 8.33 (m, 2H, Ph), 7.50 (m, 3H, Ph). Ms: (m/z) 275 (M^+).

$C_{11}H_9N_5O_2S$ (M_r 275.3)				
Calc.	C 47.99	H 3.29	N 25.44	S 11.64
Found	C 48.25	H 3.27	N 25.29	S 11.64

4-Amino-7-phenyl-8H-pyrazino[2,3-c]-1,2,6-thiadiazine 2,2-dioxide (**3**)

To a suspension of **1** (1 g, 5.65 mmol) in acetic acid (20 ml) phenylglyoxal (1 g, 7.46 mmol) was added portionwise and the mixture was refluxed for 5 h. The solution was cooled and the precipitate was filtered and recrystallized from ethanol : water to give 1.0 g (64%), mp. 288–290 °C. ^{1}H-NMR (90 MHz, DMSO-d_6): δ = 8.96 (s, 1H, H-6), 8.56 (br. s, 2H, NH_2), 8.20 (m, 2H, Ph), 7.56 (m, 3H, Ph). Ms: (m/z) 275 (M^+).

$C_{11}H_9N_5O_2S$ (M_r 275.3)				
Calc.	C 47.99	H 3.29	N 25.44	S 11.64
Found	C 48.10	H 3.40	N 25.63	S 11.37

4-Amino-6-methyl-8H-pyrazino[2,3-c]-1,2,6-thiadiazine 2,2-dioxide (**5**)

To a suspension of **1** (2 g, 11.30 mmol) in acetic acid (75 ml) kept under argon methylglyoxal dimethylacetal (1.5 g, 12.70 mmol) was added dropwise. The mixture was kept at 100 °C in an oil bath for 6 h, then brought to pH 1 by addition of concentrated HCl and stirred at room temperature for 1 h. The suspension was evaporated to dryness and the residue was treated with acetone and the solid filtered off. The solution was then evaporated to dryness and recrystallized from water to give 0.9 g (37%), mp. 282–284 °C. ^{1}H-NMR (90 MHz, DMSO-d_6): δ = 8.56 (s, 1H, H-7), 8.50 (br. s, 2H, NH_2), 2.50 (s, 3H, Me). Ms: (m/z) 213 (M^+).

$C_6H_7N_5O_2S$ (M_r 213.2)				
Calc.	C 33.80	H 3.31	N 32.84	S 15.04
Found	C 33.80	H 3.38	N 32.65	S 14.80

4-Amino-7-methyl-8H-pyrazino[2,3-c]-1,2,6-thiadiazine 2,2-dioxide (**6**)

To a suspension of **1** (1 g, 5.65 mmol) in acetic acid (50 ml) and concentrated HCl (1 ml), under argon methylglyoxal dimethylacetal (1.1 g, 9.31 mmol) was added dropwise. The mixture was kept at 100 °C in an oil bath for 6 h. The suspension was concentrated, the solid washed with acetone and recrystallized from water to give 0.5 g. (42%), mp. 310–312 °C desc. ^{1}H-NMR (90 MHz, DMSO-d_6): δ = 8.53 (br. s, 2H, NH_2), 8.33 (s, 1H, H-6), 2.50 (s, 3H, Me). Ms: (m/z) 213 (M^+).

$C_6H_7N_5O_2S$ (M_r 213.2)				
Calc.	C 33.80	H 3.31	N 32.84	S 15.04
Found	C 33.50	H 3.53	N 32.89	S 15.42

4-Amino-6-bromo-7-phenyl-1H-pyrazino[2,3-c]-1,2,6-thiadiazine 2,2-dioxide (**7**)

A solution of **3** (0.5 g, 1.82 mmol) and NBS (0.35 g, 1.97 mmol) in methanol (50 ml) was stirred at room temperature for 2 h. The solution was evaporated to dryness, treated with water (25 ml), filtered and recrystallized from water : methanol to give 0.5 g (78%),

mp. 323–325 °C. ^{1}H-NMR (90 MHz, DMSO-d_6): δ = 8.76, 8.58 (br. s, NH_2), 7.75 (m, 2H, Ph), 7.58 (m, 3H, Ph). Ms: (m/z) 355, 353 (M^+).

$C_{11}H_8N_5O_2SBr$ (M_r 354.2)

Calc.	C 37.30	H 2.28	N 19.77	S 9.05	Br 22.56
Found	C 37.05	H 2.47	N 20.01	S 8.76	Br 22.21

4-Amino-6-bromo-7-methyl-1H-pyrazino|2,3-c|-1,2,6-thiadiazine 2,2-dioxide (**8**)

A solution of **6** (0.28 g, 1.31 mmol) and NBS (0.32 g, 1.80 mmol) in methanol (20 ml) was stirred at room temperature for 6 h. The solution was evaporated to dryness, treated with water (2 ml), filtered and recrystallized from water to give 0.2 g (53%), mp. 294–296 °C. ^{1}H-NMR (90 MHz, DMSO-d_6): δ = 8.60, 8.45 (br. s, 2H, NH_2), 2.63 (s, 3H, Me). Ms: (m/z) 293, 291 (M^+).

$C_6H_6N_5O_2SBr$ (M_r 292.1)

Calc.	C 24.67	H 2.07	N 23.97	S 10.97	Br 27.35
Found	C 24.86	H 2.17	N 23.88	S 11.10	Br 27.23

4-Amino-7-methoxy-6-phenyl-1H-pyrazino|2,3-c|-1,2,6-thiadiazine 2,2-dioxide (**9**)

A solution of **2** (0.50 g, 1.82 mmol) and NBS (0.35 g, 1.97 mmol) in methanol (75 ml) was stirred at 45 °C for 10 h. The solution was evaporated to dryness, treated with water (25 ml) and filtered. The solid was dissolved in methanol, silica gel was added, and the mixture was evaporated to dryness. The resulting powder was added to a silica gel column (70–230 mesh) and compound **9** was eluted with chloroform/methanol (15:1) affording 0.2 g (36%), mp. 290–292 °C. ^{1}H-NMR (90 MHz, DMSO-d_6): δ = 8.33, 8.26 (br. s, 2H, NH_2), 8.10 (m, 2H, Ph), 7.46 (m, 3H, Ph), 4.06 (s, 3H, Me). Ms: (m/z) 305 (M^+).

$C_{12}H_{11}N_5O_3S$ (M_r 305.3)

Calc.	C 47.21	H 3.63	N 22.94	S 10.50
Found	C 47.25	H 3.66	N 22.84	S 10.19

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Pteridines
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Nucleosides, XLVII[1)]
Syntheses, Reactions and Properties of 6- and 7-p-Bromophenyllumazine N-1-Nucleosides

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Summary

Glycosylation of 6-(**2**) and 7-p-bromophenyllumazine (**3**) by the silyl method using 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose (**6**) and trimethylsilyl trifluoromethanesulfonate as catalyst proceeded regioselectively in high yields to the N-1 nucleosides (**7**, **11**). Thiations by P_4S_{10} or Lawesson's reagent led to the corresponding 4-thiolumazine ribosides (**9**, **13**). Deblocking to the free nucleosides (**8**, **10**, **12**, **14**) worked best with K_2CO_3 in MeOH. Treatment of **9** and **13** respectively by methanolic NH_3 afforded debenzoylation and displacement of the thio-function to give the corresponding isopterin N-1 ribofuranosides (**15**, **16**). 2,2′-Anhydro-nucleoside formation was achieved with **8** and **10** respectively to give **17** and **18**, which formed on acid hydrolysis the 6- and 7-substituted 1-β-D-arabinofuranosyl-lumazines **19** and **20**.

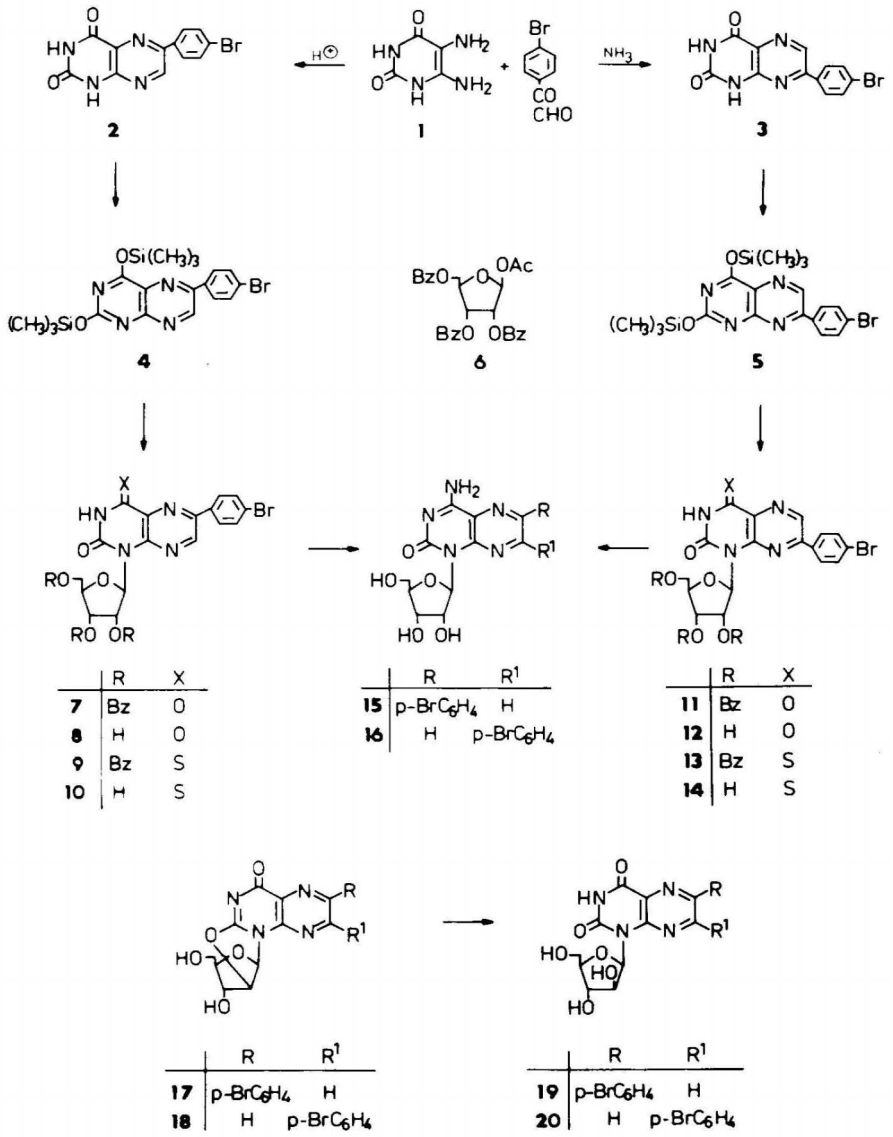
Introduction

Recent efforts to find new antivirally active compounds can be regarded as a revival of nucleoside chemistry in general. New types of nucleosides have been synthesized by modifying either the sugar moiety (1–6) or the aglycone (7–9) to achieve biological activity. The structural relationship between the lumazine N-1-nucleosides (10–14) and uridine, thymidine, and their synthetic analogues such as the antivirally active 5-iodo-(14), 5-trifluoromethyl-(15), and E-5-(2-bromovinyl)2′-deoxyuridine (16) as well as the 1-β-D-arabinofuranosylthymine (17) justifies our intention to extend our former studies in this series to newly substituted lumazine derivatives. As potentially interesting candidates appeared the 6- and 7-p-bromophenyllumazines, which have been ribosylated and modified at the aglycone and carbohydrate moieties by more or less standard reactions.

Results and Discussion

The starting materials for the glycosylation reactions have been synthesized from 5,6-uracildiamine (**1**) (18) and p-bromophenylglyoxal leading in 85% sulfuric acid solution to 6-p-bromophenyllumazine (**2**) and in an aqueous ammonia-ethanol mixture to the corresponding 7-isomer (**3**). Ribosylation was achieved by the Hilbert-Johnson-Birkofer procedure (19) converting **2** and **3** respectively first into their trimethylsilyl derivatives **4** and **5** followed by treatment with 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose (**6**) in 1,2-dichloro-ethane under trimethylsilyl trifluorosulfonate catalysis (20). These conditions guarantee a highly regioselective reaction towards N-1 substitution and afforded the 6-(**7**) and 7-p-bromophenyl-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-lumazine (**11**) in 90 and 93% isolated yield respectively. Debenzoylation of these protected ribosides worked best under moderate basic conditions, using a catalytic amount of potassium carbonate in methanol at room temperature, to give in almost quantitative yield the corresponding nucleosides **8** and **12**, respectively.

[1)] Part XLVI: Al-Masoudi, N.A.L. & Pfleiderer, W. (1990) Nucleosides & Nucleotides, *8*, 1485.



	R	X
7	Bz	O
8	H	O
9	Bz	S
10	H	S

	R	R^1
15	p-BrC_6H_4	H
16	H	p-BrC_6H_4

	R	X
11	Bz	O
12	H	O
13	Bz	S
14	H	S

	R	R^1
17	p-BrC_6H_4	H
18	H	p-BrC_6H_4

	R	R^1
19	p-BrC_6H_4	H
20	H	p-BrC_6H_4

The protected lumazine nucleosides **7** and **11** are subject to easy thiation reactions which proceeded smoothly with P_4S_{10} or Lawesson reagent in boiling dioxane to give the corresponding yellow-coloured 4-thio nucleosides **9** and **13** in excellent yields. The assignment of their structures is derived from comparisons of their UV spectra with that of 1-methyl-6,7-diphenyl-4-thiolumazine (21) as an authentic reference substance. The 4-thioxo function is light-sensitive and shows easy photooxidation back to the starting material. Debenzoylation of **9** and **13** proceeded well in MeOH with catalytic amounts of K_2CO_3 to **10** and **14**, whereas stronger nucleophiles afforded side reactions due to displacements of the thioamide function as demonstrated with conc. ammonia to form the isopterin-N-1-β-D-ribofuranosides **15** and **16**.

Furthermore the interconversion of the lumazine-N-1-β-D-ribofuranosides **8** and **12** respectively into the corresponding N-1-β-D-arabinofuranosides **19** and **20** were performed via the 2,2′-anhydro-nucleoside intermediates (**17**, **18**). Treatment of **8** and **12** with diphenyl carbonate and a catalytic amount of $NaHCO_3$ in DMF at 160 °C (12, 22) led to 6-(**17**) and 7-p-bromophenyl-2,2′-ahydro-1-β-D-arabinofurano-syllumazine (**18**), which were hydrolysed by 0.1 N H_2SO_4 in acetone to the corresponding arabinosides **19** and **20** in good yields.

Physical Properties

The structures of the newly synthesized pteridine nucleosides were assigned and proven by UV and ^{1}H-NMR spectral comparisons with known structural

Table 1. Physical data of lumazine-N-1-nucleosides

Compound	pK in H_2O	UV-Absorption spectra λ_{max} (nm)						log ε						pH	Molecular form
7		229		281			352	4.66		4.44			3.95	MeOH	o
8		[217]		281			353	[4.11]		4.41			3.92	MeOH	o
	8.27	[222]		278			351	[4.14]		4.38			3.97	6.0	o
		[220]		270			355	[4.03]		4.41			4.05	11.0	–
11		229		[274]			350	4.26		[4.10]			4.26	MeOH	o
12		231		283			351	4.32		3.97			4.32	MeOH	o
	8.30	228	[256]	286			351	4.32	[3.91]	3.88			4.38	6.0	o
		232	[254]	[282]			353	4.27	[4.07]	[3.96]			4.35	11.0	–
9		229	[256]	296			396	4.74	[3.38]	4.40			4.05	MeOH	o
10		[220]	259	290			397	[4.23]	4.48	4.49			4.05	MeOH	o
	7.47	220	256	292	[334]	[378]	397	4.20	4.28	4.37	[3.75]	[3.93]	4.11	4.0	o
		239	257	[282]	342	352	395	4.44	4.37	[4.31]	4.03	4.03	3.96	11.0	–
13		228	[269]	[294]	[322]		398	4.58	[3.96]	[3.89]	[3.83]		4.18	MeOH	o
14		[230]		294	[340]	[365]	398	[4.17]		3.91	[3.96]	[4.02]	4.07	MeOH	o
	7.95	[228]		[292]	[344]	364	399	[4.27]		[3.93]	[4.11]	4.15	4.17	5.0	o
		[236]	[260]	[287]	[344]	354	395	[4.24]	[4.15]	[4.09]	[4.25]	4.28	4.06	11.0	–
15		[232]		277			366	[4.00]		4.50			4.06	MeOH	o
16		[235]	263	[283]			362	[4.21]	4.15	[4.12]			4.31	MeOH	o
17		[215]	[250]	273			350	[4.12]	[4.20]	4.38			4.05	MeOH	o
18		232	[256]	284			347	4.33	[4.13]	4.07			4.33	MeOH	o
19		[218]		281			352	[4.17]		4.43			3.99	MeOH	o
	8.25	[220]		279	[300]		352	[4.15]		4.40	[4.07]		3.99	6.0	o
		[220]		272	[300]		356	[4.11]		4.40	[4.12]		4.05	11.0	–
20		231		284			351	4.33		4.00			4.33	MeOH	o
	8.40	229	[257]	287			352	4.29	[3.88]	3.87			4.35	6.0	o
		233	[256]	[284]			354	4.26	[4.04]	[3.96]			4.30	11.0	–

[ ] = Shoulder; o = neutral form; – = monoanion.

analogues. The small bathochromic shift of the long wavelength absorption band in the UV spectra of **8** and **12** changing from the neutral form to the monoanions is in agreement with former results (10) and indicates clearly the N-1 substitution in these nucleosides (Table 1). The introduction of a thioxo group into the 4-position causes the well-known bathochromic shift (21) of the lumazine derivatives and is also associated with an increased acidity of such molecules of about one pK_a unit in comparison to the oxo analogue. It is also noteworthy to mention that the 7-p-bromophenyllumazine derivatives exhibit, due to a stronger mesomeric interaction of the 7-substituent with the nucleus, a higher extinction than its 6-substituted counterpart. This fact can help in differentiating between 6- and 7-phenyl-substituted lumazines in general.

Other fine structural features can be depicted from the ^{1}H-NMR spectra (Table 2). The small coupling constants for 1′-H in **7**, **8**, **11**, and **12** is in agreement with the β-configuration of the glycosidic bond and a high population of the N-type conformation (23) of the ribofuranosyl moiety. Consequently, the β-arabinonucleosides (**19**, **20**) exhibit a high coupling constant on the same grounds of the Karplus relation. Furthermore, the higher degree of conformational restriction in the 2,2′-anhydro-nucleosides (**17**, **18**) is reflected in a more distinct separation of the signals of the sugar protons.

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Table 2. ^{1}H-NMR data of lumazine nucleosides

	^{1}H-NMR spectra in D_6-DMSO or $CDCl_3$* (δ-values in ppm against TMS)												
	N-H NH_2	1′-H $J_{1',2'}$	2′-H $J_{2',3'}$	3′-H $J_{3',4'}$	4′-H $J_{4',5'}$	5′-H $J_{4',5'}$	5″-H $J_{5',5''}$	2′-OH $J_{2',OH}$	3′-OH $J_{3',OH}$	5′-OH	6-Subst.	7-Subst.	Sugar Benzoyl
7*	10.40 s	7.00 d <0.5	6.30 dd 3.5	6.24 pt	4.76 m	4.88 dd 3.0	4.66 dd 12.0	–	–	–	7.3–8.0 m 4H	8.89 s	7.3–8.0 m 15H
8	12.02 s	6.52 d <0.5	4.63 m	4.25 m	3.62–3.76 m		3.49 dd 12.0	5.15 d 6.0	4.98 d 6.7	4.63 m	7.7–8.13 m		–
11*	10.2 s	7.17 d <0.5	6.34 dd 3.0	6.15 pt	4.79–4.83 m		4.66 dd 11.5	–	–	–	9.01 s	7.26–8.80 m 19H	
12	12.02 s	6.66 d <0.5	4.65 m	4.23 dd	3.61–3.77 m		3.47 dd 12.0	5.13 d 5.0	4.98 d 6.7	4.65 m	9.23 s	7.83 d, 8.15 d 4 H	–
9*	10.12 s	6.99 d 2.5	6.32 dd 7.0	6.24 pt 8.0	4.77 dd 3.0	4.88 dd 5.2	4.67 dd 11.5	–	–	–	7.3–8.0 m 4H	8.88 s	7.3–8.0 m 15H
10	13.5 s	6.55 d <0.5	4.64 m	4.25 dd 6.1	3.63–3.84 m		3.50 dd 12.0	5.18 d 5.0	4.99 d 6.7	4.64 m	7.95 d, 8.17 d 4H	8.88 s	–
13*	10.0 s	7.19 d <0.5	6.34 dd 2.9	6.15 pt	4.80–6.12 m		4.65 dd 12.5	–	–	–	9.01 s	7.30–8.1 m 4H	7.30–8.1 m 15H
14	13.5 s	6.19 d <0.5	4.58 m	4.20 pt	3.46–3.61 m		3.40 dd 11.5	5.15 d 4.9	4.97 d 6.0	4.17 m	8.70 s	7.27 d, 7.64 d	–
15	8.50 d	6.57 d 3.7	4.67 m	4.26 pt	3.76 m	3.64 dd 5.0	3.41 dd 14.0	5.04 d	4.86 d	4.67 pq	7.73 d, 8.35 d	9.38 s	–
16	8.38 d	6.67 d <0.5	4.68 m	4.24 pq	3.75 m	3.63 dd 5.5	3.44 dd 12.0	5.07 d 4.8	4.91 d 6.7	4.68 m	9.10 s	7.82 d, 8.16 d	–
17		6.72 d 5.5	5.36 d 0.5	4.48 d <0.5	4.15 d <0.5	3.28–3.39 m		–	5.95 d 4.0	4.93 pt	7.78 d, 8.15 d 4H	9.43 s	–
18		6.84 d 5.8	5.36 s	4.58 d <0.5	4.16 d <0.5	3.26–3.46 m		–	5.98 bs	4.95 pt	9.43 s	7.83 d, 8.25 d 4H	–
19	12.03 s	6.91 d 7.4	4.32 dd <0.5	4.49 m	4.26 m	3.20 d		5.30 d 5.3	5.20 d 5.5	3.46 m	7.76 d, 8.13 d 4H	9.36 s	–
20	11.75 s	7.18 d 8.0	4.38 dd <0.5	4.56 m	4.24 m	3.69 m		5.32 d 5.5	5.27 5.8	3.40 m	9.23 s	7.80 d, 8.25 d 4H	–

s = singlet; bs = broad singlet; d = doublet; dd = doublet of doublet; pt = pseudotriplet; pq = pseudoquadruplet; m = multiplet.

Experimental Part

General

UV/VIS: Perkin Elmer Spectrometer Lambda 5; λ_{max} in nm (log ε). ^{1}H-NMR: Bruker WM 250; in δ (ppm) relative to TMS. TLC: Precoated silica-gel thin-layer sheets F 1500 LS 254 and cellulose thin-layer sheets F 1440 LS 254 from Schleicher & Schüll. M. p.: Büchi apparatus, model Dr. Tottoli; no corrections. pK: determinations were performed by the spectrophotometric method (24).

6-(p-Bromophenyl)-lumazine (**2**)

In 200 ml of 85% sulphuric acid were dissolved 7.1 g (0.05 mol) of 5,6-uracil-diamine and then 10.34 g (0.05 mol) of p-bromoglyoxal hydrate added with stirring. The mixture was stirred at 65 °C for 2 h and then poured onto ice (750 g) and neutralized with conc. aqueous ammonia to pH 5. After standing over night the precipitate was filtered off (12 g) and then recrystallized from formic acid/H_2O (4/1; 600 ml) to give 10.7 g (60%) of yellowish crystals of m. p. > 300 °C. UV (MeOH): [218] (4.20); 280 (4.49); 355 (4.07).

$C_{12}H_7Br\ N_4O_2$ (M_r 366.1)			
Calc.	C 45.16	H 2.21	N 17.56
Found	C 45.00	H 2.16	N 17.43

7-(p-Bromophenyl)-lumazine (**3**)

In a mixture of 100 ml of H_2O and 100 ml of conc. aqueous ammonia were dissolved 3.55 g (0.025 mol) of 5,6-uracil-diamine (**1**) and then a solution of 5.69 g (0.03 mol) of p-bromoglyoxal hydrate in 250 ml EtOH added. The mixture was refluxed for 2 h and after cooling the dark-yellow precipitate (7.5 g) collected. Recrystallization from DMSO gave 6.79 g (56%) of yellow crystals of m. p. > 300 °C. UV (MeOH): 209 (4.42); 281 (4.06); 352 (4.40).

$C_{12}H_7Br\ N_4O_2$ (M_r 366.1)			
Calc.	C 45.16	H 2.21	N 17.56
Found	C 45.09	H 2.33	N 17.11

6-(p-Bromophenyl)-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-lumazine (**7**)

In 35 ml of hexamethyldisilazane were heated 2.62 g (7.15 mmol) of **2** and a few crystals of ammonium sulfate under reflux for 18 h. The solution was evaporated to dryness, the residue (**4**) dissolved in dry 1,2-dichloroethane (50 ml) and then a solution of 3.6 g (7.15 mmol) of 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose (**6**) in dichlorethane (20 ml) added. The mixture is stirred for 2 h at room temp. after addition of 1.5 ml of trimethylsilyl trifluoromethane sulfonate. The reaction mixture was then partitioned between CH_2Cl_2 and a dilute aqueous $NaHCO_3$ solution. The organic layer was dried (Na_2SO_4) and then evaporated to dryness. The residue gave on recrystallization from $CHCl_3$ 90% of colorless crystals of m. p. 238–239 °C.

$C_{38}H_{27}Br\ N_4O_9$ (M_r 703.6)			
Calc.	C 59.77	H 3.65	N 7.34
Found	C 59.97	H 3.76	N 7.09

6-(p-Bromophenyl)-1-β-D-ribofuranosyllumazine (**8**)

1. To dry MeOH (100 ml) were added 1.5 g (1.97 mmol) of **7** and 0.4 g of K_2CO_3. The mixture was stirred for 18 h at room temp., then evaporated to dryness, the residue dissolved in hot water and then acidified to pH 5 by dilute AcOH. The precipitate was filtered off and gave on recrystallization from MeOH 0.81 g (89%) of colorless crystals of m. p. 284 °C (decomp.).

$C_{17}H_{15}Br\ N_4O_6$ (M_r 451.2)			
Calc.	C 45.25	H 3.35	N 12.41
Found	C 45.48	H 3.40	N 12.37

2. In dry MeOH (50 ml) were dissolved 0.03 g of Na and then 0.76 g (0.1 mmol) of compound **7** added and stirred for 24 h at room temp. The reaction mixture was evaporated to a small volume (10 ml), H_2O (50 ml) added and then extracted several times with ether. The aqueous layer was treated with dilute AcOH to pH 5 to form a solid. This material was filtered and gave on recrystallization from MeOH 0.3 g (68%) of colourless crystals of m. p. 284 °C (decomp.). This product was chromatographically and spectrophotometrically identical with the previously described material.

6-(p-Bromophenyl)-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-4-thiolumazine (**9**)

1. A mixture of 1.53 g (2 mmol) and 1.3 g (2.9 mmol) of P_4S_{10} was heated under reflux in dry dioxane (50 ml) for 2 h. After cooling was filtered and then the orange filtrate evaporated to dryness. The residue was partitioned between $CHCl_3$ and brine solution, the organic extract dried over Na_2SO_4, filtered and again evaporated to a yellow solid. Recrystallization from $CHCl_3$/MeOH gave 1.4 g (92%) of yellow crystals of m. p. 253–255 °C.

$C_{38}H_{27}Br\ N_4O_8S$ (M_r 779.6)			
Calc.	C 58.54	H 3.49	N 7.18
Found	C 58.25	H 3.30	N 7.14

2. A mixture of 0.76 g (0.1 mmol) of compund **7**and 0.55 g (1.36 mmol) of Lawesson reagent in dry dioxane (30 ml) was refluxed for 48 h. After cooling the suspension was evaporated to dryness and then the residue partitioned between $CHCl_3$ and brine solution. The organic layer was dried (Na_2SO_4), filtered, evaporated and then the residue recrystallized from $CHCl_3$/MeOH to give 0.7 g (90%) of yellow crystals of m. p. 254 °C. Mixed m. p., UV and ^{1}H-NMR spectra are identical with the previously described material.

6- p-Bromophenyl)-1-β-D-ribofuranosyl-4-thiolumazine (**10**)

In dry MeOH (40 ml) was stirred 0.56 g (0.7 mmol) of **9** and 0.13 g of K_2CO_3 for 20 h at room temp. The mixture was evaporated to dryness, the residue dissolved in warm H_2O and then acidified to pH 5 with dilute AcOH to give 0.29 g (88%) of yellow crystals of m. p. 260 °C (decomp.).

$C_{17}H_{15}Br\ N_4O_5S$ (M_r 467.3)
Calc. C 43.69 H 3.18 N 11.98
Found C 43.86 H 3.41 N 11.76

7-(p-Bromophenyl)-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-lumazine (**11**)

A mixture of 2.7 g (7.0 mmol) of 6-(p-bromophenyl)-lumazine (**3**) and a few crystals of ammonium sulfate was heated in 50 ml of hexamethyldisilazane under reflux for 20 h. The solution was evaporated to dryness after cooling to a solid residue of **5**. It was dissolved in dry 1,2-dichloroethane (45 ml) and then a solution of 3.72 g (7 mmol) of 1-O-acetyl-2,3,5-tri-O-benzoyl-ribofuranose (**6**) in dichlorethane (30 ml) added and followed by the dropwise addition of the catalyst trimethylsilyl trifluoromethanesulfonate (2.5 ml). After stirring for 2 h at room temp., the solution was partitioned between chloroform (50 ml) and an aqueous solution of $NaHCO_3$ (50 ml). The organic layer was separated, dried over Na_2SO_4, filtered and evaporated to a pale yellowish residue (4.93 g). Recrystallization from chloroform/MeOH yielded 4.5 g (84%) of colorless crystals of m. p. 252–255 °C.

$C_{38}H_{27}Br\ N_4O_9$ (M_r 763.6)
Calc. C 59.77 H 3.65 N 7.34
Found C 59.65 H 3.55 N 7.29

7-(p-Bromophenyl)-1-β-D-ribofuranosyllumazine (**12**)

A solution of 1.0 g (1.3 mmol) of **11** in dry MeOH (50 ml) was stirred at room temp. after addition of 0.34 g of K_2CO_3 for 20 h. The solution was evaporated to dryness, the residue dissolved in boiling H_2O and then treated with 2 N AcOH to pH 5 to form a crystalline precipitate. Recrystallization from MeOH gave 0.56 g (93%) of colorless crystals of m. p. 256 °C (decomp.).

$C_{17}H_{15}Br\ N_4O_6 \cdot H_2O$ (M_r 469.3)
Calc. C 43.51 H 3.65 N 11.94
Found C 43.24 H 3.78 N 11.87

7-(p-Bromophenyl)-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-4-thiolumazine (**13**)

A mixture of 1.25 g (1.63 mmol) of **11** and 1.09 g (2.45 mmol) of P_4S_{10} was heated under reflux in dry dioxane (50 ml) for 1.5 h. After cooling the suspension was filtered, the filtrate evaporated to dryness, the residue dissolved in $CHCl_3$ and then treated with brine solution. The organic layer was dried (Na_2SO_4), filtered, evaporated to dryness and then the yellow residue recrystallized from $CHCl_3$/MeOH to give 1.17 g (92%) of a yellow solid of m. p. 264–266 °C.

$C_{38}H_{27}Br\ N_4O_8S$ (M_r 779.6)
Calc. C 58.54 H 3.49 N 7.18
Found C 58.39 H 3.51 N 7.16

7-(p-Bromophenyl)-1-β-D-ribofuranosyl-4-thiolumazine (**14**)

A mixture of 0.7 g (9 mmol) of **13** and 0.24 g of K_2CO_3 in dry MeOH (50 ml) was stirred at room temp. for 18 h. It was evaporated, the residue dissolved in warm H_2O and then acidified to pH 5 to give 0.38 g (90%) of yellow crystals of m. p. 253 °C (decomp.).

$C_{17}H_{15}Br\ N_4O_5S$ (M_r 467.3)
Calc. C 43.69 H 3.18 N 11.98
Found C 43.74 H 3.44 N 11.71

4-Amino-6-(p-bromophenyl)-1-β-D-ribofuranosylpteridin-2(1H)-one (**15**)

In a pressure vessel were heated 0.8 g (1 mmol of **9** in 25% methanolic ammonia (50 ml) to 100 °C for 48 h. The reaction solution was treated with charcoal, filtered, the filtrate evaporated to dryness and the residue recrystallized from MeOH to give 0.15 g (34%) of colorless crystals of m. p. 260 °C (decomp.).

$C_{17}H_{16}Br\ N_5O_5 \cdot H_2O$ (M_r 468.3)
Calc. C 43.60 H 3.87 N 14.95
Found C 43.83 H 3.58 N 15.23

4-Amino-7-(p-bromophenyl)-1-β-D-ribofuranosylpteridin-2(1H)-one (**16**)

According to the preceding procedure 0.8 g (1 mmol) of **13** yielded 0.32 g (67%) of a yellowish crystal powder of m. p. 242 °C (decomp.).

$C_{17}H_{16}Br\ N_5O_5 \cdot 1/2\ H_2O$ (M_r 459.3)

Calc.	C 44.46	H 3.73	N 15.25
Found	C 44.40	H 3.37	N 15.20

2,2'-Anhydro-6-(p-bromophenyl)-1-β-D-arabinofuranosyllumazine (**17**)

A mixture of 0.5 g (1.1 mmol) of **8**, 0.26 g (1.5 mmol) of diphenyl carbonate, and a catalytic amount of $NaHCO_3$ (10 mg) in dry DMF (15 ml) was heated to 155–160 °C for 20 min. After cooling the solvent was evaporated to dryness and the residue treated with boiling ether and then the precipitate collected. Recrystallization from MeOH yielded 0.22 g (46%) of a colorless crystal powder of m. p. 282–284 °C.

$C_{17}H_{13}Br\ N_4O_5$ (M_r 433.2)

Calc.	C 47.14	H 3.02	N 12.93
Found	C 46.80	H 2.96	N 12.37

2,2'-Anhydro-7-(p-bromophenyl)-1-β-D-arabinofuranosyllumazine (**18**)

Analogous treatment of 0.25 g (1.1 mmol) of **12** according to the preceding procedure yielded 0.21 g (42%) of colorless crystals of m. p. 265–267 °C (decomp.).

$C_{17}H_{13}Br\ N_4O_6 \cdot 1/2\ H_2O$ (M_r 442.2)

Calc.	C 46.17	H 3.19	N 12.67
	C 46.43	H 3.21	N 12.55

6-(p-Bromophenyl)-1-β-D-arabinofuranosyllumazine (**19**)

In a mixture of acetone/0.1 N H_2SO_4 (1/1; 15 ml) was heated under reflux 0.2 g (0.46 mmol) of **17** for 2 h. It was evaporated to half of the volume, chilled over night and the precipitate collected to give after recrystallization from MeOH/H_2O (1/1) 0.15 g (71%) of colorless crystals of m. p. 187 °C (decomp.).

$C_{17}H_{15}Br\ N_4O_6$ (M_r 451.2)

Calc.	C 45.25	H 3.35	N 12.42
Found	C 45.08	H 3.62	N 12.22

7-(p-Bromophenyl)-1-β-D-arabinofuranosyllumazine (**20**)

Analogous treatment of 0.2 g (0.46 mmol) of **18** according to the preceding procedure yielded 0.15 g (72%) of colorless crystals of m. p. 265–267 °C (decomp.).

$C_{17}H_{15}Br\ N_4O_6$ (M_r 451.2)

Calc.	C 45.25	H 3.35	N 12.42
Found	C 45.08	H 3.62	N 12.22

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Neopterin and Cellular Immune Activation in Active Gynaecological Cancer: Significant Correlation Between Neopterin and Circulating Number of HLA-DR Expressing Lymphocytes

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Summary

Urine concentrations of neopterin, a marker for the activation of cellular immunity, were determined concomitantly with a large panel of hematological and immunological variables on 24 women with gynaecological cancers (12 uterine cervix, 5 endometrium, 7 ovary). There were 14 women investigated at first diagnosis of carcinoma, and 10 women were studied during follow-up (evidence of disease, only palliative treatment during the preceding three months). A statistically significant correlation was found between neopterin concentrations and number of circulating non-B lymphocytes expressing HLA-DR antigen. Neopterin concentrations, numbers of HLA-DR positive cells, and numbers of circulating leukocytes were significantly higher in women studied during follow-up, when compared with women freshly diagnosed. In contrast, numbers of CD4+, CD3+, CD19+ lymphocytes and of total lymphocytes in peripheral blood were significantly lower in women studied during follow-up. The results suggest that high neopterin levels in tumor patients indicate activation of early steps of cellular immune mechanisms.

Introduction

Neopterin (6D-erythro-[1′,2′,3′-trihydroxypropyl]-pterin) is produced in large amounts by human monocytes/macrophages in response to activation with interferon gamma in vitro (1). In vivo, measurement of neopterin concentrations in body fluids, e. g., urine or serum, has been widely used as a convenient and sensitive marker for the activation of cellular immunity (2–4).

Various authors have reported raised neopterin concentrations in urine or serum of patients with malignant tumors. Moreover, high levels of neopterin at diagnosis of cancer and/or during follow-up were shown to be significantly associated with poor prognosis (5–10).

There is a considerable interest to show by more direct methods that raised neopterin concentrations in body fluids of patients with malignant disease are really due to immune activation phenomena. Here we report data cross-sectionally obtained on 24 women with gynaecological carcinomas, in whom urinary neopterin was measured concomitantly with a variety of hematological and immunological variables. By comparison of data from women with newly diagnosed cancer with those from women with malignant disease in a late phase, we attempted to describe immunological changes occurring during course of cancer.

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Patients and Methods

1. Patients

Twenty-four women were included into the study who were patients at the Department of Obstetrics and Gynaecology of the University Hospital of Innsbruck, Austria. The patients fulfilled the following enrollment criteria: histologically proven carcinoma of the uterine cervix (12 patients), of the ovary (seven patients) or of the endometrium (five patients); and the patients were not receiving any specific therapy at the time of investigation. Thus, there were 14 women in whom the malignant disease was freshly diagnosed, and 10 women who were in a late stage of their disease and were receiving only palliative therapy since at least three months. The mean age of the 14 pretherapeutic women was 64.3 yr (SD 10.5 yr) with a range from 39 to 76 yr, the 10 women with late stage disease were on average 65.3 yr old (SD 10.9 yr) with a range from 47 to 78 yr. According to the staging criteria of the International Federation of Gynaecology and Obstetrics, there were six women with stage I, eight with stage II, seven with stage III and three with stage IV. (Since the aim of this study was a first elucidation of the effect of longstanding malignant disease on neopterin and its associations with other immune parameters, and since the patients did not receive currently any tumor specific medication, the details of prior tumor specific medication of the 10 women with late disease were not analysed in detail).

2. Immunophenotyping of peripheral blood mononuclear cells (PBMC)

These analyses were performed at the Department of Internal Medicine of the University of Innsbruck. Venous blood was collected into 20 mL syringes containing heparin as anticoagulant. PBMC were obtained using a density separation (density 1.077 g/cm^3) (Percoll, Pharmacia, Uppsala, Sweden) and adjusted to a concentration of 10^7 cells per mL. For the immunophenotyping an indirect immunofluorescence assay with FITC-conjugated goat anti-mouse immunoglobulin antibody was performed.

The following monoclonal antibodies (MoAbs) were used: Leu 4 (CD3) identifying peripheral blood T lymphocytes, Leu 3a (CD4), which recognizes helper/inducer T cells, Leu 2a (CD8), reacting with antigens on suppressor/cytotoxic T cells, Leu M3 (CD14), identifying monocytes/macrophages, Leu 11 (CD16), reacting with natural killer (NK) cells, B4 (CD19), identifying B-lymphocytes, and anti-HLA-DR (Ia), reacting with major histocompatibility complex (MHC) class II antigens. The MoAbs Leu 2a, Leu 3a, Leu 4, Leu 11, Leu M3 and anti-HLA-DR (Ia) antibodies were purchased from Becton-Dickinson (Sunnyvale, California, U. S. A.), and B4 from Coulter Immunology (Hialeah, Florida, U. S. A.).

The immunofluorescence analyses were made using a fluorescence activated cell sorter (FACStar, Becton-Dickinson).

For the present investigation, only the lymphocyte fraction of peripheral blood cells was evaluated. To obtain the number of immunologically relevant HLA-DR$^+$ lymphocytes, the number of B lymphocytes as assessed with the antibody B4 (CD19) was subtracted from the entire number of positive cells. Thus, "HLA-DR$^+$ lymphocytes" in this paper always refers to this corrected number.

3. Determination of neopterin

Concomitantly with collection of blood, first morning urine specimens were obtained from the patients. These were protected from daylight using a tin-foil and stored at -20 °C until analysis. The determination of neopterin was performed at the Institute for Medical Chemistry and Biochemistry of the University of Innsbruck. For the analysis, an optimized and automated high performance liquid chromatography technique on reversed phase was used which has been described earlier (3). Neopterin was quantitated by its native fluorescence at 353 nm excitation and 438 nm emission wavelengths. To correct for physiological variations of urine concentrations, values were related to creatinine which was determined in the same chromatographic run, using its UV absorption at 235 nm. Neopterin concentrations are therefore given in µmol per mol creatinine.

4. Normal values

In order to put the observed neopterin data and the results of the immunologic variables in perspective, we report the normal ranges used. For urinary neopterin, the upper limit of normal is slightly age- and sex-dependent and varies between 230 to 250 µmol neopterin per mol creatinine for women of the age categories under interest. The normal ranges for the immunological variables are: number of CD3+ cells, 800 to 2500 per µL; number of CD4+ cells, 430 to 1200 per µL; number of CD8+ cells, 180 to 870 per µL; CD4+/CD8+ T cell ratio, 1 to 2.3; percentage of CD16+ cells, 4 to 26%; percentage of CD19+ cells, 4 to 23%.

5. Statistical procedures

Due to the preliminary character of the study, P-values below 0.10 were assumed to indicate "statistical significance". Statistical tests used were the Mann-Whitney U-test for comparison of data obtained in pretherapeutic women vs late stage women, linear correlation analysis and Spearman rank correlation analysis.

Results

Table 1 summarizes the observations. As can be seen from the selected percentiles shown, for several variables the distributions were distinctly asymmetric.

Since it was the aim of the study to analyse possible effects of longstanding malignancy on immune parameters, data obtained on patients freshly diagnosed were compared with those obtained on women with long-standing disease by the Mann-Whitney test. The results are shown in Figure 1 (variables for which the level of statistical significance exceeded 0.10 are not shown). Two groups of variables were seen: neopterin concentrations, numbers of HLA-DR$^+$ non-B cells and numbers of leukocytes were significantly higher in women with long-standing disease than in patients at primary diagnosis. In contrast, numbers of total and lymphocyte subgroups positive for CD4 and CD3 were significantly lower in women with long-standing disease.

The data were further analysed by correlation analysis. By linear correlation analysis, neopterin showed the strongest correlation with the number of HLA-DR$^+$ cells ($r = .638$; $P = 0.0005$). Several other variables were also significantly correlated with neopterin ($P < .10$); these were in order to decreasing strength of correlation, numbers of thrombocytes ($r = .408$, $P = .047$), of CD3$^+$ cells ($r = -.377$, $P = .069$) and of CD4$^+$ cells ($r = -.363$, $P = .081$). Slightly different results were obtained by Spearman's rank corre-

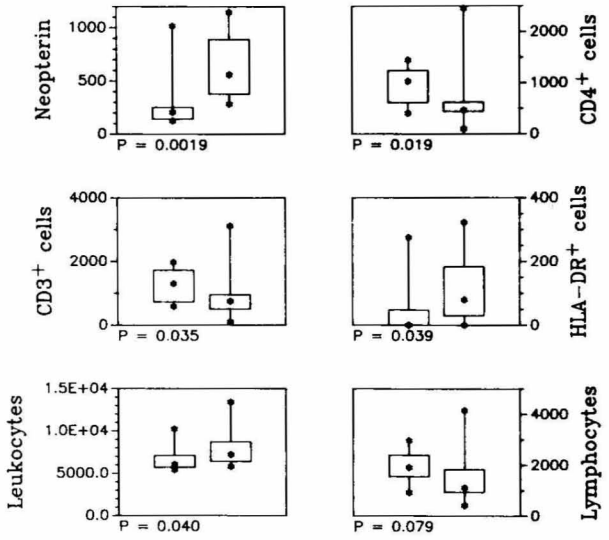

Figure 1. Box plots showing the distributions of data observed in women at first diagnosis (left box for each variable) and in women with evidence of disease during follow-up (right box for each variable).
In the center of each box, the star indicates the median value, the lower and upper limits of the box denote the 25th and 75th percentiles, and the vertical lines indicate the range of observations. For units, see Table 1.

Table 1. Distributions of observed values of the studied variables

Variable	Minimum value	Maximum value	Percentile		
			25th	50th	75th
a) Peripheral whole blood					
Hemoglobin[a)]	91	151	109	119	129
Leukocytes[b)]	5400	13400	5800	6400	8200
Lymphocytes[b)]	420	4154	1060	1700	2330
Monocytes[b)]	155	1005	365	515	645
Thrombocytes[c)]	184	513	222	288	329
Neopterin[d)]	124	1146	184	287	741
b) Peripheral blood lymphocyte fraction					
CD3$^+$ cells[b)]	109	3115	655	955	1650
CD4$^+$ cells[b)]	100	2450	465	620	1100
CD8$^+$ cells[b)]	88	708	260	410	560
CD16$^+$ cells[b)]	0	1083	90	167	253
CD19$^+$ cells[b)]	66	390	111	169	232
CD4$^+$/CD8$^+$ ratio	0.6	4.2	1.4	2.1	2.8
HLA-DR$^+$ cells[b)e)]	0	323	0	32	107

[a)] g/L; [b)] μL^{-1}; [c)] nL^{-1}; [d)] μmol/mol creatinine; [e)] B cells were excluded (see Methods).

lation analysis, pointing to the non-Gaussian character of the distributions: for neopterin, significant correlations were found with numbers of HLA-DR$^+$ cells ($R_s = .517$, $P = .013$), CD4$^+$ cells ($R_s = -.502$, $P = .015$), CD3$^+$ cells ($R_s = -.475$, $P = .024$), and thrombocytes ($R_s = .345$, $P = .098$). Figure 2 shows the results obtained for neopterin plotted versus number of HLA-DR$^+$ cells.

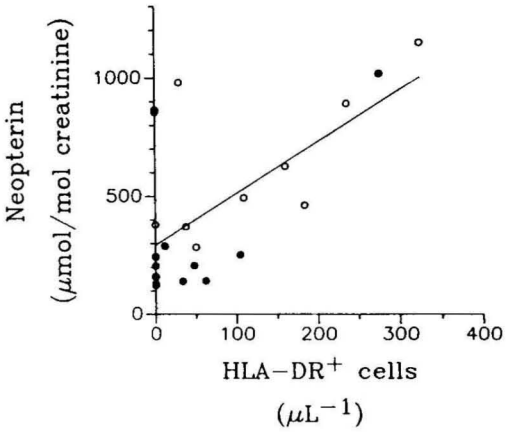

Figure 2. Scatter plot showing the positive correlation between numbers of HLA-DR$^+$ cells (CD19$^+$ cells excluded) and urinary neopterin concentrations found in women with gynecological neoplasias at first diagnosis (solid circles) and with evidence of tumor during follow-up (open circles).

Discussion

Raised concentrations of neopterin are frequently reported in patients with malignancies (3). The finding that this substance is produced by interferon gamma activated human monocytes/macrophages (1) but not by tumor cells themselves pointed to a preactivation state of cell-mediated immunity in cancer patients. The consistent observation by various research groups of a significant association of high neopterin concentrations and poor prognosis (5–10) is of particular interest not only for patient's management but also for a better founded immunobiological understanding of the interactions taking place between the immune system and a growing tumor.

Our results contribute to this growing body of evidence for the presence of immune activation in cancer patients. Whereas the numbers of circulating lymphocytes were lower in women with long-standing disease than in those studied pretherapeutically, the concentrations of neopterin in urine and the number of non-B lymphocytes expressing HLA-DR antigen and blood cells were significantly higher. Neopterin and number of HLA-DR$^+$ non-B lymphocytes, both being indicators of immune activation and very likely of endogenous interferon gamma production, were significantly and positively correlated with each other.

Our data, and previous data on neopterin in cancer patients, indicate that certain immune mechanisms are activated. Indeed, this reasoning is strongly supported by observations in patients with hematological neoplasias showing significantly higher concentrations of endogenous interferon gamma and a significant and positive correlation between interferon gamma and neopterin levels (11). Neopterin increases do not, however, provide information whether or not the immune response can eventually eliminate the source of immune activation, e. g., tumor cells.

It is well known for a long time that patients with malignant tumors show diminished responsiveness against recall-antigens, and that immune cells derived from these patients are incapable to respond adequately against antigenic or mitogenic stimuli in vitro. Also, in vitro production of interferon gamma by T cells from cancer patients is usually decreased. Thus, there appears to exist an inverse relationship between endogenous interferon gamma production which appears to be raised and the reduced capacity of in vitro production of this lymphokine in response to antigenic stimulation. We suggest that mechanisms induced by persistently activated immune mechanisms and particularly persistent cytokine production may contribute to immunodeficiency: It is known that immune response to antigenic stimulation requires an appropriate amount of interferon gamma (12–14) and interleukin 2 (15, 16). Both too low and too high doses inhibit development of a proper immune response. The first possibility (too low) is suggested by the observation that anti-interferon gamma antibodies interfere with alloantigen- or tumor antigen-driven immune response (12, 13, 15). The second situation (too high) is compatible with data by our group showing that simultaneous stimulation of human peripheral blood mononuclear cells with purified protein derivative (PPD) of mycobacteria and exposure to high concentration of interferon gamma diminishes subsequent response to PPD but not to interleukin-2 (17). Interleukin-2 is required for the autocrine proliferation of T cells, but interferon gamma is synthesized by T cells independently of proliferation (18). Thus, antigenic stimulation may induce production and release of interferon gamma while later steps of T cell activation and particularly interleukin-2 synthesis may be inhibited.

Besides the consistent notion of high neopterin concentrations in patients with malignant tumors, a strong argument for the presence of immune activation are our findings on the increase of the number of HLA-DR$^+$ non-B cells which is significantly associated with an increase of neopterin levels. Much evidence has been accrued to indicate that interferon

gamma is able to regulate class II histocompatibility antigen expression on many different cell types, including macrophages (19–21).

Besides these effects, persistent immune activation could also be at least partly responsible for some of the other changes in immune parameters that have been observed in this study. For example, cytokines may affect the expression of erythroid progenitors and possibly other hematopoietic precursors (22). This may be partly the cause for, e. g., the decrease of total lymphocytes and lymphocyte subsets. This may be true even for variables for which we were unable to detect a significant correlation with neopterin, because the effects may be small and masked by other causes: looking at the average values, our results would be compatible with such effects.

In sum, the results of our study which of course can be regarded only as preliminary, support the view that in patients with active malignant disease early events of cellular immune mechanisms may be activated. The positive correlation between neopterin concentrations and numbers of HLA-DR$^+$ cells renders substantial weight to the conclusions drawn from earlier studies that raised neopterin concentrations in tumor patients are not a product of the tumor cells, but rather reflect activation of certain cellular immune mechanisms.

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