6-AZAURIDINE-5′-URONIC ACID AND RELATED COMPOUNDS: SYNTHESIS AND BINDING TO PROTEINS*

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6-Azauridine-5′-uronic acid (*V*) was prepared by the catalytic oxidation of 2′,3′-O-isopropylidene--6-azauridine (*II*) with sodium periodate in the presence of ruthenium trichloride and the subsequent acidic hydrolysis. By reaction of compound *II* with sodium chloroacetate and acidic hydrolysis, there was prepared 6-azauridine-5′-O-acetic acid (*VII*). 6-Azauridine-2′(3′)-O-acetic acid (*IX*) was obtained analogously from 5′-O-trityl-6-azauridine (*III*). Condensation of compounds *V*, *VII*, and *IX* with bovine gamma-globulin and human serumalbumin according to the method of mixed anhydrides afforded conjugates containing up to 11% of covalently bound nucleoside derivative.

The purpose of the preparation of conjugates containing various immunosuppressants covalently bound to protein antigenes consists in the effort to obtain an increased and highly specific immunosuppressant activity. Some conjugates containing 6-mercaptopurine derivatives bound to bovine gamma-globulin and human serumalbumin have been recently reported[1-3]; investigations on the biological activity of these conjugates are in progress. The immunosuppressant activity is exhibited *inter alia* by some nucleosides or corresponding heterocyclic bases[4]; since also 6-azauracil[5] and 6-azauridine[6-10] show immunosuppressant properties, an attempt was made to prepare such 6-azauridine derivatives which would made possible binding to proteins. It must be born in mind in this connection that the presence of additional groups should not interfere with the characteristic chemical and physicochemical properties of 6-azauridine. In this respect, the carboxylic acid derivatives appeared as the most suitable because of the easy condensation with proteins by the procedure of mixed anhydrides[11]. Some analogous nucleoside derivatives have been earlier prepared and used for the same purpose[12-15]. On the other hand, the other routes applied to bindings of nucleosides to proteins such as through the diazonium derivatives[16] or through dialdehydes (obtained from ribosides by the periodate

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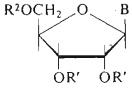
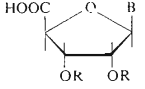
oxidation[17]) are not suitable for the present purpose since the structure of the substance to be bound to the protein is modified to a too great extent.

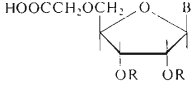
The simplest route, namely, the preparation of 5′-O-succinyl-6-azauridine and its binding to proteins, was soon abandoned because of the low stability of esters of this type and the high inclination of conjugates to hydrolysis. In another route, 6-azauridine-5′-uronic acid (*V*) was used. The preparation of nucleoside 5′-uronic acids requires such oxidants which would be sufficiently active to oxidize the primary hydroxylic group but which would not attack other parts of the molecule, especially the heterocyclic moiety or the nucleoside bond. The catalytic oxidation with air or oxygen over platinum dioxide is widely used in this respect; this method has also been examined[19] in the case of 6-azauridine but the results are not encouraging. Some other oxidants[20–23] are destructive with 6-azauridine. On the other hand, the recently reported[24] method for oxidation of primary hydroxylic groups in sugar



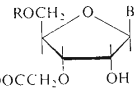
I, $R^1 = R^2 = H$
II, R^1, $R^1 = (CH_3)_2C$, $R^2 = H$
III, $R^1 = H$, $R^2 = (C_6H_5)_3C$



IV, R, R = $(CH_3)_2C$
V, R = H

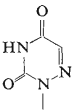


VI, R, R = $(CH_3)_2C$
VII, R = H



VIII, R = $(C_6H_5)_3C$
IX, R = H

(or the 2′-isomer)

In formulae *I*–*IX*, B = 

derivatives is smooth and affords the required result also in the case of 6-azauridine derivatives. Thus, a high yield of 2′,3′-O-isopropylidene-6-azauridine-5′-uronic acid (*IV*) is obtained by reaction of 2′,3′-O-isopropylidene-6-azauridine (*II*) with sodium periodate in aqueous acetone and in the presence of ruthenium trichloride. The isopropylidene group of compound *IV* is extraordinarily stable. A reflux in 80% aque-

ous acetic acid for 5 h is required for its removal while 30 minutes are quite sufficient with other nucleoside derivatives. The acid *V* was isolated in the form of the lithium salt and its structure was confirmed on the basis of analysis, chromatography,

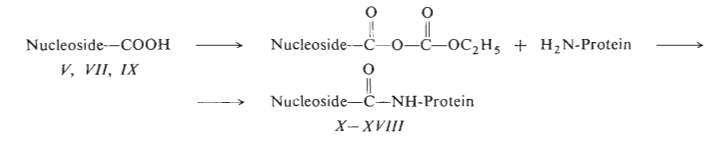


TABLE I
Conjugates

6-Azauridine derivative	Protein[a]	Conjugate	Content[b], %	Molar ratio[c]	Yield[d], %
V	HSA	*X*	5·4	16	32·0
	HSA	*XI*	10·8	34	34·0
	BGG	*XII*	4·7	33	22·0
VII	HSA	*XIII*	4·7	14	28·0
	HSA	*XIV*	9·5	30	30·0
	BGG	*XV*	4·5	31	20·6
IX	HSA	*XVI*	6·6	20	40·0
	HSA	*XVII*	10·9	35	35·0
	BGG	*XVIII*	3·6	25	16·6

[a] HSA, human serumalbumin; BGG, bovine gamma-globulin; [b] covalently bound 6-azauracil, as determined spectrophotometrically; [c] number of covalently bound derivative residues per one molecule of the protein; [d] determined spectrophotometrically for the starting amounts of the acids *V* or *VII* or *IX*.

TABLE II
Paper Chromatography and Electrophoresis

Compound	*I*	*II*	*IV*	*V*	*VI*	*VII*	*IX*
R_F in S_1	0·48	0·75	0·57	0·28	0·60	0·31	0·18
E_{Up}[a] in E_1	0·52	—	—	1·12	—	1·10	1·10
E_{Up}[a] in E_2	—	—	—	1·00	—	0·83	1·00

[a] The mobility refers to uridine 3′-phosphate.

electrophoresis, and ultraviolet spectrum (almost identical with that of 6-azauridine *I* both in the acidic and the alkaline region, *cf.*[25]); the structure is also in accordance with the course of the synthesis.

In the acid *V* or its conjugates with proteins, some changes may occur in the conformation of the sugar moiety due to the chemical modification of the sugar portion. For this reason, we have attempted the preparation of 6-azauridine derivatives with an ethereally bound acetic acid residue. The preparation of such ethers by reaction of 6-azauridine derivatives with ethyl bromoacetate in the presence of a base (triethylamine, sodium hydride) is accompanied by a considerable decomposition of reactants. On the other hand, the earlier reported[25] treatment of the sodium nucleoside alkoxide with sodium chloroacetate proved more advantageous. Dimethyl sulfoxide appears as the most suitable solvent (in dimethylformamide, the yields are lower). Thus, 2',3'-isopropylidene-6-azauridine (*II*) in dimethyl sulfoxide was first converted by the action of sodium hydride to the corresponding sodium alkoxide (with the use of two equivalents of sodium hydride, there is formed the sodium salt of both the heterocyclic and sugar moiety at position 5') which was then treated with sodium chloroacetate at room temperature to afford the protected derivative *VI*. Deblocking of compound *VI* by acidic hydrolysis gave the free 6-azauridine-5'-O-acetic acid (*VII*). Neither N-substituted nor O,N-disubstituted derivatives were formed, obviously due to the high nucleophilicity of the alkoxide anion. The structure of compounds *VI* or *VII* (isolated as the lithium salt) may be inferred from analysis, chromatography, and electrophoresis (the N-substituted derivative would be less acidic than the O-derivative). The mobility of compound *VII* corresponds to two negative charges at pH 7·5 (the N^3—H function of 6-azauracil and the carboxylate anion). Moreover, the UV spectrum (λ_{max} 260 nm at pH 2 and λ_{max} 254 nm at pH 12) exhibits the unchanged chromophoric system of 6-azauracil[26].

The isomeric derivative *IX* was prepared from 5'-O-trityl-6-azauridine (*III*) under analogous conditions, *i.e.*, with the use of two equivalents of sodium hydride. The protecting trityl group was then removed under acidic conditions and the thus-obtained compound *IX* isolated by chromatography on DEAE-cellulose. The product *IX* was chromatographically as well as electrophoretically homogeneous (its mobility was similar to that of the 5'-isomer *VII*). The structure *IX* was confirmed analogously to that of compound *VII*. As it may be seen from the reaction Scheme, compound *IX* represents a mixture of 2'- and 3'-isomers (these isomers are difficult to separate). Similarly to compound *II*, the trityl derivative *III* does not afford the N-isomer in the reaction with sodium chloroacetate. Some disubstituted derivatives are formed when a greater excess of both sodium hydride and sodium chloroacetate is used. Even under such conditions, the $O^{2',3'}$-disubstituted product predominates over the N,O-disubstituted derivative.

Conjugates of the above 6-azauridine derivatives with proteins were prepared according to the method of mixed anhydrides[11]. Compounds *V*, *VII*, and *IX* are

highly soluble in dioxane which is used as medium in the heterogeneous reaction step. To exclude side reactions (inter- and intramolecular) of the above polyfunctional molecules under the conditions usual in condensations with proteins, some model experiments were performed and their products analyzed by chromatography. There was formed a small amount of contaminants which are cleaved under the dialysis conditions, *i.e.*, at pH 10·5. Reaction of the *in situ* prepared mixed anhydrides of compounds *V*, *VII*, and *IX* with ethoxycarbonyl chloride, with bovine gamma-globulin and human serumalbumin was effected in aqueous dioxane at a weakly alkaline pH value. The reaction mixture was dialyzed against alkaline ammonium hydrogen carbonate buffer solution (3 days), the resulting mixture analyzed by gel filtration on Sephadex G-25 and freeze-dried in the absence of low-molecular contaminants.

The amount of the nucleoside derivative bound to the protein was determined spectrophotometrically after subtraction of the own absorption of the protein (at the wavelength of the 6-azauridine absorption maximum, 260 nm). For yields and amounts of the covalently bound nucleoside derivative see Table I. Yields referred to the starting compound are 28–40% with human serumalbumin and 16–22% with bovine gamma-globulin. There were obtained conjugates containing 4·7–10·9%, *i.e.*, 14–35 nucleoside residues per one molecule of serumalbumin and 3·6–4·7%, *i.e.*, 26–33 nucleoside residues per the molecule of gamma-globulin. The higher is the substitution, the greater is the hypsochromic shift of the absorption maximum with respect to the starting protein (about 280 nm). With conjugates containing a high amount of 6-azauracil, the maxima lie at 262–265 nm, *i.e.*, in the region of 6-azauridine absorption maximum. It was found that the conjugates are homogeneous on gel electrophoresis (agarose as carrier) and travel to the anode.

EXPERIMENTAL

Unless stated otherwise, the solutions were taken down on a rotatory evaporator at 40°C/15 Torr and the analytical samples were dried over phosphorus pentoxide at 0.1 Torr.

Materials and Methods

Human serumalbumin and bovine gamma-globulin were preparations of Forschungsinstitut für Impfstoffe, Dessau, German Democratic Republic. The UV spectra were measured on a SF 8 recording spectrophotometer and the quantitative runs were effected on a Spectromom 204 apparatus. With derivative *V*, *VII*, and *IX* in aqueous solution, the value $\varepsilon_{260} = 6000$ was used.

Descending chromatography was performed on paper Whatman No 1 in the solvent system S_1, 2-propanol–conc. aqueous ammonia–water (7 : 1 : 2). Electrophoresis was carried out on paper Whatman No 3 MM by the reported technique[27] at 40 V/cm for 45 min in buffer solutions E_1, 0·1M triethylammonium hydrogen carbonate (pH 7·5), and E_2, 0·05M sodium hydrogen citrate (pH 3·5). Spots were detected by viewing under UV light (Chromatolite) or, in the case of *cis*-diols, by the reported technique[28]. For the R_F values and electrophoretical mobilities see Table II. Gel electrophoresis was performed on a VEB Carl Zeiss, Jena apparatus (301 E Statron as source and Ag/AgCl electrodes) at 90 V and 50 mA. As the carrier there was used 0·8% agarose gel

(Agarosa, Serva, Heidelberg, German Federal Republic). Sodium barbiturate — sodium acetate (pH 8·6, $I = 0{\cdot}1$) was used as buffer. The electrophoresis was performed for 90 min. After the fixation, the plates were dried and treated first with Amidoblack 10B and then 25% aqueous acetic acid. The conjugates and the starting proteins were visible as blue spots. Gel filtration was performed on a 1·5 × 30 cm column of Sephadex G-50 ("Fine"; Pharmacia, Sweden) in 0·01M borate buffer solution (pH 9·2), the 3 ml fractions being taken.

6-Azauridine-5'-uronic Acid (*V*)

To a solution of 2',3'-O-isopropylidene-6-azauridine[29] (*II*; 2·14 g; 7·5 mmol) in 70% aqueous acetone (60 ml) there was added sodium periodate (6·50 g) and 5% aqueous ruthenium trichloride (0·12 ml), and the whole was stirred at room temperature for 4 h. After this period of time, the starting compound *II* is absent as shown by electrophoresis in the buffer solution E_1. Ethanol (20 ml) was then added, the mixture stirred at room temperature for 1 h, diluted with acetone (50 ml), the solid filtered off, and washed with acetone. The filtrate and washings were combined and evaporated under diminished pressure. The residue was dissolved in water (5 ml), the solution applied to a column (150 ml) of Dowex 50 X 8 (H^+) ion exchange resin, and the column eluted with water. The UV-absorbing eluate was adjusted with barium hydroxide to pH 8, concentrated under diminished pressure to the volume of about 5 ml, the concentrate filtered, and the filtrate precipitated with excess ethanol. After 1 h, the precipitate was collected by centrifugation, washed with acetone, and dissolved in water (10 ml). The aqueous solution was applied to a column (100 ml) of Dowex 50 X 8 (H^+) ion exchange resin. The column was eluted with water and the UV-absorbing eluate evaporated under diminished pressure. The residue was refluxed in 80% aqueous acetic acid (100 ml) for 5 h, the mixture evaporated, the residue coevaporated with three 20 ml portions of water, and the final residue dissolved in water (10 ml). The aqueous solution was adjusted with 10% aqueous lithium hydroxide to pH 7·5, filtered through Celite, and the material on the filter washed with water. The filtrate and washings were combined and evaporated under diminished pressure. The residue was coevaporated with two portions of methanol and finally dissolved in hot methanol (10 ml). The solution was slowly cooled down to the incipient crystallisation which was completed with ethanol. After 1 h, the mixture was diluted with acetone (200 ml) and kept at room temperature overnight. The solid was collected by centrifugation and washed with acetone and ether. Yield, 1·75 g (77·5%) of the lithium salt of compound *V* (dried over phosphorus pentoxide at 60°C/0·1 Torr) homogeneous on chromatography and electrophoresis. For $C_8H_8LiN_3O_7.2\,H_2O$ (301·1) calculated: 31·90% C, 4·02% H, 13·96% N; found: 32.29% C, 4.05% H, 14·11% N. UV spectrum: λ_{max} 260 nm (pH 2); λ_{max} 254 (pH 12).

6-Azauridine-5'-O-acetic Acid (*VII*)

To a solution of compound[29] *II* (2·85 g; 10 mmol) in dimethyl sulfoxide (50 ml) there was added sodium hydride (0·50 g; 20 mmol) and the mixture stirred at room temperature for 15 min under exclusion of atmospheric moisture. Dry sodium chloroacetate (1·28 g; 11 mmol) was then added and the whole stirred at room temperature for 4 h. The mixture was kept at room temperature for 2 days, diluted with ethanol (10 ml) and 80% aqueous acetic acid (300 ml), the whole refluxed for 2 h, and evaporated under diminished pressure. The residual dimethyl sulfoxide was removed by a successive coevaporation with dimethylformamide and toluene at 65°C/0·1 Torr. The final residue was dissolved in water (10 ml) and the aqueous solution applied to a column (150 ml) of Dowex 50 X 8 (H^+) ion exchange resin. The UV-absorbing fraction was eluted with water, the eluate adjusted with 10% aqueous lithium hydroxide to pH 7·5, and evaporated under diminished pressure. The residue was dissolved in methanol (10 ml) and the solution precipitated

with 500 ml of 1 : 1 ethanol–acetone. The precipitate was collected by centrifugation, washed with acetone, and dissolved in water (10 ml). The aqueous solution was applied to a column (150 ml) of Dowex 50 X 8 (H^+) ion exchange resin, the product eluted with water, the eluate adjusted with saturated aqueous barium hydroxide to pH 8, and concentrated under diminished pressure to the volume of about 10 ml. The concentrate was precipitated with ethanol-acetone as above. The precipitate of the barium salt of the product was transformed to the lithium salt as described above and precipitated from methanol with ethanol–acetone. The solid was collected by centrifugation, washed with acetone and ether, and dried. Yield, 2·48 g (68%) of the lithium salt of compound *VII*, homogeneous on chromatography and electrophoresis. Content (spectrophotometrically): 87·5%. For $C_{10}H_{12}LiN_3O_8 . 3 H_2O$ (363·2) calculated: 33·07% C, 5·00% H, 11·57% N; found: 32·81% C, 5·36% H, 11·35% N. UV spectrum: λ_{max} 260 nm (pH 2); λ_{max} 254 nm (pH 12).

6-Azauridine-2'(3')-O-acetic Acid (*IX*)

A mixture of 5'-O-trityl-6-azauridine[30] (*III*; 5·0 g; 10 mmol) and sodium hydride (0·80 g) in dimethyl sulfoxide (25 ml) was stirred for 15 min under exclusion of atmospheric moisture, treated with sodium chloroacetate (1.3 g) and the whole stirred at room temperature overnight. The semi-solid mixture was then diluted with ethanol (10 ml), stirred for 30 min, neutralised with glacial acetic acid (5 ml), and diluted with 80% aqueous acetic acid. The whole was refluxed for 2 h, concentrated to a small volume under diminished pressure, the concentrate diluted with water (300 ml), and extracted with three 100 ml portions of ether. The aqueous phase was evaporated at 50°C/15 Torr and the residue coevaporated with three 50 ml portions of water and one 100 ml portion of dilute aqueous ammonia under the same conditions. The final residue was dissolved in water (50 ml) and the aqueous solution applied to a 80 × 4 cm column of DEAE-cellulose (Cellex D, standard capacity, HCO_3^- form). The column was eluted with a linear gradient of triethylammonium hydrogen carbonate pH 7·5 (3 l of water in the mixing chamber, 3 l of 0·2M buffer solution in the reservoir). The rate was 3 ml per min and the fractions were taken in 10 min intervals. The course of the elution was checked on a Uvicord apparatus (LKB, Sweden). The product was in the 0·08—0·15M buffer fraction. The eluate was evaporated under diminished pressure and the volatile buffer was removed by coevaporation with methanol. The final residue was dissolved in water (20 ml) and the aqueous solution applied to a column (100 ml) of Dowex 50 X 8 (Li^+ cycle) ion exchange resin. The column was eluted with water, the eluate evaporated, the residue dissolved in water (10 ml), and the aqueous solution precipitated successively with ethanol (100 ml), acetone (100 ml), and ether (200 ml). The lithium salt of compound *IX* was collected with suction, washed with ether, and dried under diminished pressure; yield, 1·9 g (53·6%); content (spectrophotometrically), 91%. For $C_{10}H_{12}LiN_3O_8$ (309·2) calculated: 13·60% N; found: 12·25% N. UV spectrum: λ_{max} 260 nm (pH 2); λ_{max} 254 nm (pH 12).

Preparation of the HSA Conjugate *X*

A solution of the lithium salt of compound *V* (0·090 g; 0·3 mmol) in water (3 ml) was applied to a column of Dowex 50 X 8 (H^+) ion exchange resin (10 ml) and the elution performed with water (50 ml). The eluate was evaporated under diminished pressure, the residue coevaporated with two portions of dioxane, dried over phosphorus pentoxide overnight, and dissolved in fresh dioxane (3 ml). The solution was treated with tri-n-butylamine (0·075 ml; 0·3 mmol) and cooled down to 10°C. Ethoxycarbonyl chloride (0·029 ml) was then added and the whole kept at 10°C for 20 min under exclusion of atmospheric moisture. The resulting mixture was then added in one portion under stirring and cooling (0°C) to a solution of human serumalbumin (HSA) (0·41 g;

0·006 mmol) in 50% aqueous dioxane (22 ml) and 1M-NaOH (0·42 ml). After 4 h, the mixture was dialyzed for 3 days against 0·01M ammonium carbonate pH 10·5 (a solution of 10 g of ammonium carbonate in 5 l of water is adjusted with 25% aqueous ammonia to pH 10·5); the buffer solution was replaced three times. In order to determine the presence of the free acid *V*, a portion of the mixture corresponding to 3·2 mg of the original HSA was gel-filtered (see above). Forty fractions of the eluate were measured at 260 nm. After the determination of the content at 260 nm, the whole mixture was freeze-dried.

Preparation of the HSA Conjugate *XI*

The reaction was performed with 0·3 mmol of compound *V*; conversion to the mixed anhydride was performed analogously to the conjugate *X*, the mixture was added to a solution of HSA (0·21 g; 0·003 mmol) in 40% aqueous dioxane (10 ml) and 1M-NaOH (0·42 ml), and the whole was processed analogously to the preparation of the conjugate *X*.

Preparation of the BGG Conjugate *XII*

Compound V (0·15 mmol) was converted to the mixed anhydride analogously to the preparation of the conjugate *X* and then added to a solution of bovine gamma-globulin (BGG) (0·16 g; 0·001 mmol) in 0·9% aqueous sodium chloride (10 ml) and 1M-NaOH (0·14 ml). The mixture was processed analogously to the preparation of the conjugate *X*. After dialysis, the mixture was subjected to centrifugation and freeze-drying. The conjugates *XIII*—*XVIII* were prepared similarly. For the results see Table I.

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