PREPARATION OF CONJUGATES OF URIDINE WITH PROTEINS BY THE IMIDO ESTER CONDENSATION METHOD*

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Reaction of 5'-O-*p*-toluenesulfonyl-2',3'-O-isopropylideneuridine (*I*) with sodium 4-cyanophenoxide afforded 2',3'-O-isopropylidene-5'-O-(4-cyanophenyl)uridine (*II*) which was converted by acid hydrolysis into 5'-O-(4-cyanophenyl)uridine (*IIIa*). Acid-catalyzed addition of ethanol to compound *IIIa* gave the imido ester hydrochloride *IIIb* which on reaction with ammonia or ethylamine was transformed into the amidine derivatives *IIIc* and *IIId*. Compound *IIIb* reacted with human serum albumine or bovine gamma-globuline at pH 9·2 to give protein conjugates with uridine, bound covalently by an amidine bond (*IIIe,f*).

Within the framework of investigations of protein conjugates with nucleobases and biologically active nucleosides, we described methods of attaching nucleosides of cytostatic, virostatic and immunosuppressive activity[1,2] to protein antigenes[3-6]. Various bonding methods, used for this purpose, were all based on activation of a carboxylate type ligand by *e.g.* mixed anhydrides, active esters or carbodiimides. The common feature of these conjugates is a marked change in physical properties caused by loss of basic amino groups of (preponderantly) the lysine moieties which on condensation with the ligand are transformed into amide groups or into substituted thioureas[3,5,6]. This disadvantage can be removed by using ligands of the imido ester type which react with basic amino groups to form amidine bonds, leaving thus the charge distribution in the protein unchanged within the whole physiological region of pH. Another advantage of this method, already used by us previously for preparation of conjugates of uracil and 5-halogenouracils[4], are mild conditions of the condensation; this method has therefore been used for the chemical modifications of proteins very often[7-12].

The aim of this study was to elaborate a method for preparation of nucleoside derivatives, carrying a group capable of imido ester condensation and to check the condensation with proteins. We chose uridine as model nucleoside; as a ligand, potentially capable of active imido ester formation, we chose the 4-cyanophenyl group which can easily be transformed into an imido ester according to ref.[13].

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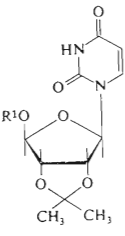
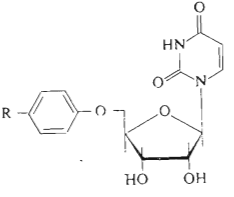
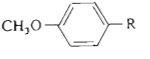
Reaction of 5'-O-*p*-toluenesulfonyl-2',3'-O-isopropylideneuridine (*I*) with sodium 4-cyanophenoxide in dimethylformamide at elevated temperature afforded the desired 5'-O-(4-cyanophenyl)-2',3'-O-isopropylideneuridine (*II*); its structure was proved unequivocally by IR spectrum which exhibited a nitrile band at 2233 cm^{-1} and bands due to an aromatic ring as well as bands corresponding to the NH and C=O groups of the uracil system. Acid hydrolysis of compound *II* with acetic acid afforded 5'-O-(4-cyanophenyl)uridine (*IIIa*) in high yield. In our case, it was not possible to convert the cyano group into the imido ester group using the relatively mild method of Baker and Erickson[14] because the reaction with methyl iodide would lead to substitution at the N^3 position of uracil. Therefore we chose the more





III



IV

I, R^1 = *p*-toluenesulfonyl
II, R^1 = *p*-cyanophenyl

In formulae *III*, *IV*: *a*, R = —C≡N

b, R = —C(=$NH_2^{(+)}$)—OC_2H_5 $Cl^{(-)}$

c, R = —C(=$NH_2^{(+)}$)—NH_2 $Cl^{(-)}$

d, R = —C(=$^{(+)}NH—C_2H_5$)—NH_2 $Cl^{(-)}$

e, R = —C(=$^{(+)}NH—HSA$)—NH_2 $Cl^{(-)}$

f, R = —C(=$^{(+)}NH—BGG$)—NH_2 $Cl^{(-)}$

HSA ... human serum albumine, BGG ... bovine gamma globuline residue.

drastic method of Pinner[13], *i.e.* treatment of compound *IIIa* with ethanolic hydrogen chloride in dioxane. At 5°C the optimum range of hydrogen chloride concentration is very narrow: under different conditions the reaction time was too long and the conversion low or substantial decomposition of the mixture took place. Upon partial precipitation with ether the reaction mixture afforded a good yield of the imido ester hydrochloride *IIIb* which served as starting material for further condensation reactions.

In order to obtain comparison samples for spectrophotometric characterization and at the same time to prove the structure of the compound *IIIb*, we performed its reaction with ammonia and ethylamine. The compound reacted smoothly in methanol at elevated temperature to afford the amidine hydrochlorides *IIIc* and *IIId* which gave satisfactory elemental analyses.

The UV spectral data of the compounds *IIIc* and *IIId* are important for the characterization of conjugates of proteins with compound *IIIa* (estimation of the bonded ligand). Both the compounds contain two isolated chromophores: the N^1-substituted uracil moiety and the *p*-alkoxyphenylamidine or *p*-alkoxyphenyl-N-ethylamidine grouping. In order to determine parameters of the second group of chromophores, we prepared by the above-mentioned method 4-methoxyphenylamidine hydrochloride (*IVc*) and 4-methoxyphenyl-N-ethylamidine hydrochloride (*IVd*) from 4-cyanoanisole (*IVa*) *via* the imido ester hydrochloride *IVb*. The UV spectra of *IIIc,d* and *IVc,d* (Table I) prove that the spectral curve for compounds *IIIc,d* represents a superposition of the uridine (N^1-substituted uracil) chromophore and chromophore of the compound *IVc* or *IVd*. The lower extinction coefficient of compounds *IIIc* and *IIId* at pH 8·7 can be ascribed to the hypochromic effect of uracil at this pH which we already observed *e.g.* for 1-carboxymethyluracil[5]. For the above reason, we determined the extent of bonding of *IIIb* to the protein, using the spectroscopic characteristics of compound *IIId* (N-substituted amidine).

Condensation of the imido ester hydrochloride *IIIb* with proteins was performed under conditions which, according to our previous experience, proved to be optimal[4], *i.e.* in a borate buffer of pH 9·2. A solution of compound *IIIb* in dimethyl sulfoxide was added to a solution of the protein in the mentioned buffer and after 24 hours the mixture was worked up by gel filtration. The high molecular weight portion was collected and content of the covalently bound ligand was determined spectrophotometrically. The yields of human serum albumin conjugates ranged between 57–63% (with 2·2–7·4% of the bound ligand), yields of conjugates of bovine gamma-globulin were 49–58% (bound ligand content 1·7–6·0%) (Table II). The substitution degree increased with increasing amount of the compound *IIIb* used in the reaction. With highly substituted conjugates we observed a hypsochromic shift of the absorption maximum and a greater band extinction in comparison with the starting protein. We can thus conclude that 5'-O-(4-cyanophenyl) ethers of nucleosides are suitable starting compounds in the condensation of nucleosides with proteins.

EXPERIMENTAL

Melting points were determined on a Boetius block. Unless stated otherwise, the solutions were evaporated at 40°C/2 kPa and the compounds were dried at 13 Pa over phosphorus pentoxide. Human serum albumin was a preparation from Forschungsinstitut für Impfstoffe (Dessau, GDR), bovine gamma-globulin was purchased from Ferak Co (West Berlin).

TABLE I
Ultraviolet Absorption Spectra

Compound	Water, pH 7			Ammonium carbonate[a,b] pH 8·7			0·01M-NaOH[b]		
	λ_{max}	ε_{max}	λ_{min}	λ_{max}	ε_{max}	λ_{min}	λ_{max}	ε_{max}	λ_{min}
IIIa	248	27 000[c]	224	—	—	—	—	—	—
IVa	248	18 000	220	—	—	—	—	—	—
IIIc	260	22 400	228	260	21 200	230	254	18 700	230
IVc	261	13 300	229	261	13 300	229	252	12 100	223
IIId	255	23 900	227	255	22 600	229	250	21 300	225
IVd	255	14 200	227	255	14 200	227	249	12 850	224

[a] The buffer was prepared by dissolving 10·0 g of ammonium carbonate in 1 l of water and adjusting the pH to 8·7 with ammonia; [b] because of lability of amidines in alkaline media the solutions were measured immediately after their preparation; [c] inflexion at 265 nm.

TABLE II
Preparation and Properties of Conjugates *IIIe,f*

Conjugate	Reaction ratio *IIIb*: protein	Yield %	Bonded Urd[a] %	mol Urd per mol protein
IIIe	10	68·3	2·18	6·3
	20	58·6	3·97	11·7
	40	57·0	7·44	22·8
IIIf	20	58·1	1·74	11·6
	40	51·0	3·01	20·4
	80	48·7	5·60	39·0

[a] The calculation is based on the uridine moiety (m.w. = 243·3).

Thin-layer chromatography was performed on Silufol UV_{254} plates (Kavalier, Czechoslovakia) in the system S1 ethyl acetate–cyclohexane (1 : 1), S2 ethyl acetate, S3 dioxane–benzene (1 : 2), or on Merck plates in the system S4 ethyl acetate–acetic acid–water (6 : 2 : 3, upper layer). Spots were detected in the UV light, in system S4 with iodine solution. The R_F values are given in Table III. Preparative gel chromatography was carried out on a column (100 × 2 cm) of Sephadex G 50 coarse (Pharmacie, Sweden) in an ammonium carbonate buffer, pH 8·7 (Table I); elution rate 1·2 ml/min, 3 ml fractions. The elution was monitored by a Uvicord instrument. UV spectra were measured on a Specord UV-VIS spectrophotometer (Karl Zeiss, Jena, GDR), quantitative determinations were performed on a Spectromom 203 instrument (Hungarian Optical Works, Budapest, Hungary). IR spectra were taken in chloroform on a Zeiss UR 10 spectrophotometer. Covalently bound uridine in conjugates *IIIe, f* was determined at 255 nm in an ammonium carbonate buffer, pH 8·7, the absorbance of non-modified protein solution of the same concentration being subtracted. The used extinction coefficient value was $\varepsilon_{255} = 22\,600$.

5′-O-(4-Cyanophenyl)-2′,3′-O-isopropylideneuridine (*II*)

A solution of 1M sodium methoxide (20 ml) was added to a solution of 4-cyanophenol[15] (2·40 g; 20 mmol) in ethanol (50 ml) and the solution was taken down *in vacuo*. The residue was twice codistilled with ethanol and dried overnight *in vacuo* over phosphorus pentoxide. A mixture of thus-prepared sodium 4-cyanophenoxide (0·60 g; 5 mmol), 5′-O-*p*-toluenesulfonyl-2′,3′-O-isopropylideneuridine[16] (I) (2·20 g; 5 mmol) and dimethylformamide (25 ml) was heated to 145°C for 6 h with exclusion of moisture. After evaporation in *vacuo*, the residue was dissolved in chloroform and washed successively (50 ml portions) with water, 5% sodium hydrogen carbonate,

TABLE III

Thin-layer Chromatography on Silica Gel Plates (R_F values)

Compound	S1	S2	S3	S4
II	0·16	0·76	0·43	0·86
IIIa	0	0·25	0·05	0·74
IIIb	0	0	0	0·35
IIIc	0	0	0	0·36
IIId	0	0	0	0·32
IVa	0·87	0·95	0·80	0·95
IVb	0	0	0	0·56
IVc	0	0	0	0·55
IVd	0	0	0	0·52
Uracil	0	0·13	0·05	0·62
2′,3′-O-Isopropylidene-uridine	0·05	0·36	0·17	—
4-Cyanophenol	0·70	0·95	0·54	—

and again with water, dried over magnesium sulfate and taken down *in vacuo*. The remaining syrupy residue was chromatographed on 3 plates (40 × 15 × 0·3 cm) of silica gel, containing a fluorescence indicator (Service Laboratories of Institute of Organic Chemistry and Biochemistry, Prague), in the system benzene–ethyl acetate (1 : 1). The UV-absorbing band (R_F 0·40) was eluted with methanol and the eluate taken down *in vacuo*, affording 60% of the compound *II*, m.p. 198—199°C (50% aqueous methanol). For $C_{19}H_{19}N_3O_6$ (385·4) calculated: 59·20% C, 4·96% H, 10·90% N; found: 58·76% C, 5·08% H, 10·69% N. IR-spectrum (chloroform): ν(C≡N) 2233 cm^{-1}, ν(ring) 1608, 1580, 1513 cm^{-1}, ν(NH) 3396 cm^{-1}, 3185 cm^{-1} (bonded), ν(C=O) 1697 cm^{-1} (1720 cm^{-1} ch), $\delta S(CH_3)$ 1381 cm^{-1} (unresolved doublet).

5′-O-(4-Cyanophenyl)uridine (*IIIa*)

A solution of compound *II* (1·93 g; 5 mmol) in 80% acetic acid (35 ml) was heated to 90°C for 3 h. After evaporation, the residue was codistilled three times with water (80 ml) and then with ethanol. Crystallization from ethanol afforded the compound *IIIa*, m.p. 190—191°C; yield 85%. For $C_{16}H_{15}N_3O_6$ (345·3) calculated: 55·65% C, 4·38% H, 12·17% N; found: 55·83% C, 4·19% H, 12·30% N.

5′-O-(4-Ethoxyiminocarbonylphenyl)uridine Hydrochloride (*IIIb*)

A solution of hydrogen chloride in ethanol (concentration 10M; 7 ml) was added at 8°C to a solution of *IIIa* (0·69 g 2 mmol) in dioxane (11 ml) and the mixture was set aside at 5°C for 8 days. Ether (80 ml) was added and the solid, separated after 2 h was collected on filter, washed with ether and light petroleum and dried *in vacuo*. The product was dissolved with stirring in a minimum amount of methanol at room temperature, the solution was filtered and ether was added until the solution became turbid. After standing for 2 h at 5°C, the product was filtered, washed with ether and dried *in vacuo*, m.p. 128—136°C; yield of *IIIb* 80%. For $C_{18}H_{22}ClN_3O_7$ (427·8) calculated: 50·53% C, 5·18% H, 9·82% N; found: 50·11% C, 5·31% H, 9·61% N.

5′-O-(4-Amidinophenyl)uridine Hydrochloride (*IIIc*)

A saturated methanolic ammonia solution (0·5 ml) was added to a solution of *IIIb* (0·17 g; 0·4 mmol) in ethanol (4 ml) and the mixture was warmed to 60°C for 3 h. After standing overnight at 5°C, the product was filtered, washed with a small amount of ethanol and dried *in vacuo*. The mother liquor on addition of ether afforded a further small amount of the same product, m.p. 179—182°C (water); yield 75%, For $C_{26}H_{19}ClN_4O_6$ (398·8) calculated: 48·18% C, 4·80% H, 14·05% N; found: 48·28% C, 4·99% H, 14·00% N.

5′-O-(4-Ethylamidinophenyl)uridine Hydrochloride (*IIId*)

Ethylamine (0·15 ml) was added to a solution of compound *IIIb* (0·17 g; 0·4 mmol) in ethanol (4 ml) and the mixture was heated to 60°C for 3 h. After standing overnight in a refrigerator the product was filtered, washed with little ethanol and crystallized from hot water (acetone added until turbid), affording compound *IIId*, m.p. 228—230°C (dec.); yield 70%. For $C_{18}H_{23}$·ClN_4O_6 (426·9) calculated: 50·65% C, 5·43% H, 13·13% N; found: 50·31% C, 5·37% H, 12·98% N.

4-Ethoxyiminocarbonylanisole Hydrochloride (*IVb*)

A solution of hydrogen chloride in ethanol (concentration 10M; 5·5 ml) was added at 8°C to a solution of compound *IVa* (1·06 g; 8 mmol) in dioxane (5·5 ml). The mixture was worked up as de-

scribed for the compound *IIIb*, affording *IVb*, m.p. 122—126°C (reported[17] m.p. 130°C); yield 90%.

4-Amidinoanisole Hydrochloride (*IVc*)

A saturated solution of ammonia in methanol (3 ml) was added to a solution of compound *IVb* (0·43 g; 2 mmol) in ethanol (25 ml) and the mixture was kept at 60°C for 3 h. After concentration to half of the original volume *in vacuo*, the product was precipitated with an excess of ether, filtered and dissolved in boiling ethanol. After cooling, ether was added and the compound *IVc* was collected; m.p. 218—222°C (reported[17] m.p. 220°C); yield 75%.

4-N-Ethylamidinoanisole Hydrochloride (*IVd*)

Ethylamine (0·75 ml) was added to a solution of compound *IVb* (0·43 g; 2 mmol) in ethanol (25 ml) and the mixture was warmed to 60°C for 3 h. The work-up procedure was the same as described for the compound *IVc*, affording the compound *IVd*, m.p. 85—89°C, in an 85% yield. For $C_{10}H_{15}ClN_2O$ (214·7) calculated: 55·94% C, 7·04% H, 13·05% N; found: 56·06% C, 7·01% H, 12·93% N.

Preparation of the Conjugates *IIIe*

A solution of the compound *IIIb* (4·3 mg — 10 μmol, 8·6 mg — 20 μmol, or 17·1 mg — 40 μmol) in dimethyl sulfoxide (0·5 ml in each case) was added to a solution of human serum albumin (69 mg; 1 μmol) in 0·2M borate buffer, pH 9·2 (2·0 ml in each case) and the mixture was set aside for 24 h at room temperature. A portion (1 ml) of the reaction mixture was subjected to gel chromatography. The high molecular weight portion (fraction 16—25) was diluted with the same buffer (in all cases up to the same volume) and the UV spectrum was taken. The conjugates were isolated by freeze-drying. The yields and properties of the conjugates *IIIe* are given in Table II.

Preparation of Conjugates *IIIf*

The title compounds were prepared analogously as described for *IIIe*, using solutions of bovine gamma-globulin (80 mg; 0·5 μmol) in 0·2M borate buffer, pH 9·2, (2 ml) and solutions of the compound *IIIb* (according to Table II). Yields and properties of the conjugates *IIIf* are listed in Table II.

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