SYNTHESIS OF CONJUGATES OF 5'-O-CARBOXYMETHYL--5-HALOGENO-2'-DEOXYURIDINES AND 5'-O-CARBOXYMETHYL-5-HALOGENOURIDINES WITH PROTEINS*

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Received June 26th, 1979

5'-O-Carboxymethyl-2'-deoxyuridine (*Ia*), its 5-fluoro, 5-bromo and 5-iodo derivatives *Ib—Id*, and 5'-O-carboxymethyluridine (*IIa*) and its 5-halogeno derivatives *IIb—IId*, on reaction with isobutyl chloroformate and tri-n-butylamine afforded mixed anhydrides *IIIa—IIId* and *IVa—IVd*. Condensation of *III* and *IV* with human serum albumin or bovine gamma-globulin at pH 8·0 gave conjugates *Va—Vd* to *VIIIa—VIIId*, the yield of the covalently bound haptene being 28 to 43%.

Some 5-halogenouracils and their N_1-substituted derivatives – including ribo- and 2'-deoxyribonucleosides – exhibit cytostatic, virostatic and immunosuppressive properties[1,2]. We tried therefore to synthesize protein conjugates, containing such compounds in a covalently bonded form with the aim to obtain potent, specifically acting, immunosuppressants[3,4]. Towards this end we worked out syntheses of N_1--carboxymethyl-5-halogenouracils[2], 5'-O-carboxymethyl-5-halogeno-2'-deoxyuridines (*I*) and 5'-O-carboxymethyl-5-halogenouridines (*II*) (ref.[2,5]), which, *via* their activated 4-nitrophenyl esters, reacted with proteins to give the corresponding conjugates[6]. Although this method gives satisfactory yields and a high degree of the haptene bonding, it requires isolation of active esters and purification of the products by gel filtration, since *p*-nitrophenol and contingent other side products formed in the reaction, cannot be removed by dialysis. Of other methods of bonding to proteins, there was described condensation of 5-fluoro-2'-deoxyuridine with albumin using a soluble carbodiimide[7]. This method, however, is accompanied by intramolecular reactions of the protein, leading to cross-linking and lower solubility of the conjugates[8].

The mentioned drawbacks could be removed by an alternative procedure, based on use of mixed anhydrides (ref.[9–11]). The reaction conditions used in this method exclude also side reactions of the 5-halogenouracil nucleus with the reagent, *i.e.*

* Part XXVI in the series Immunosuppressant Antigene Conjugates; Part XXV: This Journal *44*, 3023 (1979).

TABLE I

UV-Spectra of Compounds *I* (ε values in parentheses, wavelength in nm).

Compound	λ	Water pH 7	Ammonium carbonate buffer, pH 8·7	0·01M-NaOH pH 12
Ia	Max	260 (10 000)	260 (8 700)	260 (7 500)
	Min	231	234	243
Ib	Max	269 (8 900)	267 (6 800)	267 (6 800)
	Min	234	248	248
Ic	Max	279 (9 240)	276 (6 600)	276 (6 600)
	Min	242	252	252
Id	Max	288 (7 000)	282 (5 380)	280 (5 300)
	Min	247	253	253

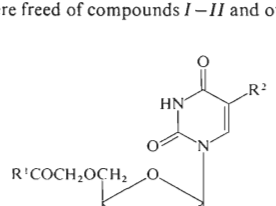
TABLE II

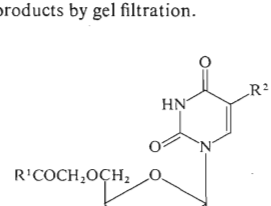
Properties of the Conjugates *V*—*VIII*

Product	Excess of haptene[a]	Yield %	Haptene content, %[b]	Substitution degree[c]
Va	25	43·2	3·43	10·8
Va	50	38·0	5·89	19·0
Vc	50	32·1	6·67	16·1
Vd	50	35·2	8·61	17·6
VIa	50	29·8	4·98	14·9
VIb	50	42·0	7·33	21·0
VIc	50	30·9	6·86	15·5
VId	50	32·1	7·62	16·1
VIIa	25	41·9	2·89	21·0
VIIb	50	36·2	4·89	36·2
VIIc	50	36·9	6·60	36·9
VIId	50	32·8	7·03	32·8
VIIIa	50	31·0	4·48	31·0
VIIIb	50	39·6	6·14	39·6
VIIIc	25	34·0	3·62	17·0
VIIId	50	28·1	5·84	28·1

[a] The amount of *III* or *IV* (μmol) used in the reaction with 1 μmol of human serum albumin or with 0·5 μmol of bovine gamma-globulin; [b] calculated for the residue of *I* or *II*; [c] mol of the haptene, bound to 1 mol of protein.

ester of chloroformic acid. The reaction was performed with *in situ* prepared tri-n-butylammonium salts of compounds *I* and *II*, which were condensed with an equimolar amount of isobutyl chloroformate in dioxane at 10°C. The obtained anhydrides *III* and *IV* were then treated directly with human serum albumin or bovine gamma-globulin in a phosphate buffer, pH 8·0. It is likely, that the ω-amino groups of lysine moieties are bonded by stable amide bonds. The formed conjugates *V*–*VIII* were freed of compounds *I*–*II* and other side products by gel filtration.





I	R^1 = OH	*II*
III	R^1 = OCOO-i-C_4H_9	*IV*
V	R^1 = NH—HSA	*VI*
VII	R^1 = NH–BGG	*VIII*

In formulae *I*–*VIII*, *a* R^2 = H, *b* R^2 = F, *c* R^2 = Br, *d* R^2 = I; HSA human serum albumine residue, BGG bovine γ-globuline residue.

The content of the covalently bound haptene and the reaction yield were determined either directly, *i.e.* by measuring the difference between the extinction coefficient of the conjugate and that of protein solution of the same concentration, or indirectly by determination of the amount of unreacted compounds *I* or *II* in the low-molecular weight fraction[12]. In the calculations we used the molar extinction coefficients, given in Table I. Both methods gave practically identical results which are listed in Table II. The yields ranged from 28% to 43% and the conjugates contained 15–40% (molar) of the haptene. The absorption maximum of the conjugate *Va* shifted hypsochromically with greater extent of substitution; this effect was less expressive in the case of *Vb*. All the prepared conjugates were homogeneous according to agarose gel electrophoresis and travelled to anode.

EXPERIMENTAL

The solutions were evaporated at 40°C. Human serum albumin was purchased from Forschungsinstitut für Impstoffe, Dessau (G. D. R.), bovine gamma-globulin from Ferak (West Berlin). Preparative gel chromatography was carried out on Sephadex G-50 coarse (Pharmacia, Sweden),

on a 100 × 2 cm column, in an ammonium carbonate buffer, pH 8·7 (10 g of ammonium carbonate in 5 l of water, pH adjusted with ammonia), elution rate 1·2 ml/min, 3 ml fractions. The course of elution was followed by a Uvicord (LKB, Sweden) instrument. UV spectra were measured on a Specord UV-VIS (Zeiss, Jena, G.D.R.) spectrometer, quantitative analyses were performed on a Spectromom 203 (Hungarian Optical Works, Budapest) instrument. Gel electrophoresis was carried out according to ref.[13].

Preparation of Anhydrides *III* and *IV*

A solution of compound *I* or *II* (ref.[2,5]) (150 μmol) in water (2 ml) was applied on a column (20 ml) of Dowex 50X8 (H^+ form) and eluted with water (Uvicord). The eluate was taken down *in vacuo* and codistilled with dioxane (3 × 5 ml). The residue was dissolved in dioxane (2·5 ml) and tri-n-butylamine (38 μl; 150 μmol) was added. After cooling to 10°C, the mixture was treated with isobutyl chloroformate (20 μl; 150 μmol) and kept at 10°C for 20 min. The solution was made up to 3 ml with dioxane and employed in the condensation reactions.

Preparation of Conjugates *V* and *VI*

An aliquot amount of the above-prepared solution of the mixed anhydride *III* or *IV* (25 and 50 μmol, respectively) was added at 10°C to a solution of human serum albumin (69 mg; 1 μmol) in 0·1M phosphate buffer, pH 8·0 (4 ml). The mixture was set aside for 1 h at 10°C and then at room temperature overnight. An aliquot (1 ml) was applied on a Sephadex column and eluted under above-mentioned conditions. High-molecular fractions (12—21) were combined, made up to an appropriate volume and used for measurements. Low-molecular UV-absorbing fractions (29—43) were pooled and used for the determination of compounds *I* and *II*. Lyophilisation of the high-molecular portion afforded the solid conjugates *V* and *VI* (Table II).

Preparation of Conjugates *VII* and *VIII*

An aliquot of the reagent *III* or *IV* (25 or 50 μmol, respectively) was added at 10°C to a solution of bovine gamma-globulin (80 mg; 0·5 μmol) in 0·01M phosphate buffer, pH 8·0 (4 ml) and the mixture was worked up in the same manner as described for the conjugates *V* and *VI*. The results are given in Table II.

Determination of Bonded Haptene Content in the Conjugates *V*—*VIII*

a) The measurements were performed with the solution of the conjugate (*vide supra*) in an ammonium carbonate buffer, pH 8·7, which was compared with a protein solution of the same concentration at the wavelength of the absorption maximum of the haptene, using extinction coefficients given in Table I.

b) The content of *I* or *II* in the eluate from the low-molecular weight fraction (*vide supra*) was determined using the values in Table I. The difference between the starting amount and the amount found in this fraction corresponded to the bound haptene.

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Translated by M. Tichý.