SYNTHESIS OF CONJUGATES OF 5-HALOURACILS WITH PROTEINS USING ACTIVATED ESTERS*

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Received September 4th, 1978

1-Carboxymethyluracil (*Ia*) and its 5-fluoro (*Ib*), 5-bromo (*Ic*) and 5-iodo (*Id*) derivatives were transformed into the *p*-nitrophenyl esters *IIa*—*IId* by reaction with *p*-nitrophenol in the presence of N,N'-dicyclohexylcarbodiimide. Reaction of these compounds with ammonia or ε-aminocaproic acid afforded the corresponding 1-aminocarbonylmethyluracil (*IIIa*—*d*) and 1-N-(5-carboxypentyl)aminocarbonylmethyluracil (*IVa*—*d*) derivatives. Reaction of compounds *II* with human serum albumin and bovine γ-globulin at pH 9·2 gave high yields of conjugates of the type *V* and *VI*, respectively, containing 10—50 uracil derivative moieties bound to the protein molecule.

Since 5-halouracil derivatives, including N-substituted compounds, ribo- and 2-deoxyribofuranosides, exhibit cytostatic, virostatic and immunosuppressive properties[1,2] we tried in our previous studies[3,4] to synthesize conjugates of some of these compounds covalently bonded to the protein antigen with the aim to prepare potent and, if possible, specific immunosuppressants. Derivatives of 5-fluoro-, 5-chloro- or 5-bromouracil with isothiocyanate[5] or imido ester structure[6] were used earlier for the binding to proteins. These methods, however, cannot be used for binding of 5-iodouracil compounds since the attempted preparation of activated compounds is accompanied by elimination of the iodine[5,6]. Also the method of mixed anhydrides encounters difficulties caused by limited solubility of 1-carboxymethyl derivatives[4,7] *I* in water and dioxane. We decided therefore to study as a general method of preparing these conjugates the condensation of *p*-nitrophenyl esters of 1-carboxymethyluracil and its 5-halogeno derivatives (*II*) with human serum albumin and bovine γ-globulin.

Active esters were used already several times for binding compounds to proteins[8—10], their advantage being a lower tendency to side reactions, either of the

* Part XXI in the series Immunosuppressant Antigene Conjugates; Part XX: Pharmazie *32*, 750 (1977).

haptene itself or of the protein molecule; these side reactions accompany often the *in situ* formation of active intermediates (*e.g.* by the method of mixed anhydrides).

In this work, the *p*-nitrophenyl esters *II* were prepared by condensation of substituted 1-carboxymethyluracils (*I*) with *p*-nitrophenol in dimethylformamide in the presence of N,N'-dicyclohexylcarbodiimide at room temperature. After removal of the solvent and N,N'-dicyclohexylurea, the *p*-nitrophenyl esters *II* were isolated in high yields by crystallisation from ethanol; they are sufficiently stable at room temperature and in the dark.

In order to gain information on the reactivity of the compounds *II* and on spectroscopic properties of the N-substituted amides derived from the compounds *I*, we studied as model reactions the transformations of compounds *II* by the action of ammonia or ε-aminocaproic acid. Reaction of compounds *IIa–d* with methanolic ammonia at room temperature afforded the corresponding 1-aminocarbonylmethyl derivatives *III* in high yields. On the other hand, work with ε-aminocaproic acid encountered difficulties, connected either with the instability of compounds *II* in aqueous and aqueous-ethanolic solutions, or with the insolubility of ε-aminocaproic acid in solvents in which compounds *II* are stable and soluble. Thus, *e.g.*, after addition of tri-n-butylamine, ε-aminocaproic acid forms even in dimethylformamide a two-phase system which upon addition of compounds *II* did not afford the desired products. This problem was solved by using tetraethylammonium ε-aminocaproate which is soluble in dimethylformamide and reacts with compounds

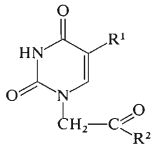
TABLE I

Ultraviolet Spectra of Substituted Uracil-1-ylacetic Acids and Their Derivatives (λ_{max}, nm)

Compound	Water		Ammonium carbonate pH 8·7[a]		0·01M-NaOH	
	λ_{max}	ε_{max}	λ_{max}	ε_{max}	λ_{max}	ε_{max}
Ia	269	10 100	269	9 450	267	8 900
IVa	266	10 000	266	9 650	264	8 500
Ib	276	9 750	274	8 250	273	7 900
IVb	272	9 600	271	8 150	270	7 850
Ic	285	8 600	282	7 400	281	7 100
IVc	282	8 650	280	7 500	279	7 100
Id	293	7 600	288	6 800	284	6 000
IVd	291	7 600	283	6 250	280	6 100

[a] *Cf.* Methods.

II to give 1-N-(5-carboxypentyl)aminocarbonylmethyl derivatives of the type *IV*. The prepared compounds *III* and *IV* were analytically pure. The UV spectral bands of the N-substituted amides *IV* in neutral and alkaline solutions (Table I) exhibit a hypsochromic shift of 2–5 nm as compared with the maxima of the compounds *I*, and a small hyperchromic effect. From the observed values of molar extinction coefficients the haptene content in the conjugates with proteins was calculated.



I, R^2 = OH
II, R^2 = O—$C_6H_4NO_2$—*p*
III, R^2 = NH_2
IV, R^2 = $NH(CH_2)_5COOH$
V, R^2 = NH—HSA
VI, R^2 = NH—BGG

In formulae *I*–*VI*: *a* R^1 = H, *b* R^1 = F, *c* R^1 = Br, *d* R^1 = I; in formulae *V* and *VI* HSA human serum albumin residue, BGG bovine gamma-globulin residue.

We can assume that the lysine ω-amino groups of the proteins attack preferentially the strongly electrophilic carbonyl reaction center of the activated esters *II* under formation of N-substituted amides. In order to find optimum conditions for the condensation of compounds *II* with proteins we studied the reaction of compound *IIa* with human serum albumin in 0·2M borate buffers at pH 7·0–10·5. We found that the conversion of the uracil derivative to the conjugate increases with increasing pH value: 40% at pH 7. 51% at pH 8, 73% at pH 9·2 and 76% at pH 10·5. At pH 7·0 and 8·0 the compound *IIa* dissolved only during the reaction whereas at pH 9·2 and 10·5 the reaction mixture was homogeneous from the beginning. Since at the last two pH values the yields differ only insignificantly, we performed all further condensations of compounds *II* with proteins in a 0·2M borate buffer at pH 9·2 in order to minimize possible undesirable changes of the protein due to the alkaline medium.

The preparation of conjugates of the type *V* and *VI* consists in addition of a solution of the compound *II* in dimethyl sulfoxide to a solution of the protein in 0·2M borate buffer (pH 9·2), followed by incubation at room temperature. Since the arising 4-nitrophenol and the product of hydrolysis of the compound *II* (compound *I*) can be removed only slowly by dialysis of the reaction mixture, the high-molecular portion was isolated by gel filtration. The content of covalently bonded uracil deriva-

tive (and thus also the yield of the reaction) was determined spectrophotometrically at the absorption maximum wavelength of the corresponding uracil derivative in an ammonium carbonate buffer (pH 8·7) after subtraction of the protein absorption, using molar extinction coefficients of the corresponding compounds *IV* (Table I). Since in some cases[11] the molar extinction coefficient of the conjugate differed somewhat from the sum of the corresponding values for the protein and haptene (or the compound *IV*) – a fact which possibly could be attributed to intramolecular interactions – the content of the bonded haptene in the conjugate was determined also by analysis for *p*-nitrophenol and compound *I*, contained in the low-molecular weight portion after gel filtration.

The conversion of compounds *II* to covalently bonded uracil derivatives in the conjugates with human serum albumin was in the range 59–78%, for conjugates

TABLE II
Yields and Properties of Protein Conjugates

Compound	Mol *II* per mol protein applied	Yield, %	Content, %[a]	Molar ratio[b]
Va	10	74·0	1·18	7·4
Va	20	78·4	2·54	15·7
Va	40	73·1	4·48	29·2
Va	80	62·8	7·47	50·2
Vb	20	58·8	2·16	11·8
Vb	40	65·0	4·63	26·0
Vc	20	62·9	3·35	12·6
Vc	40	73·0	7·45	29·2
Vd	20	76·4	5·01	15·3
Vd	40	63·1	7·95	25·2
VIa	10	75·1	0·52	7·6
VIa	20	71·0	0·98	14·2
VIa	40	67·1	1·84	26·8
VIa	80	65·0	3·51	52·0
VIb	40	54·9	1·76	22·0
VIb	80	59·8	3·78	47·8
VIc	40	65·3	3·06	26·2
VIc	80	69·0	6·36	55·2
VId	40	71·0	4·12	28·4
VId	80	65·2	7·46	52·2

[a] Calculated for covalently bound substituted uracil residue ($C_4H_2N_2O_2R$, R = H, Hal); [b] number of covalently bound substituted uracil residues per protein molecule.

with bovine γ-globulin the conversion was 55–75%. The properties of the prepared conjugates are given in Table II: the serum albumin conjugates contain (according to the character of the hapten) 1·2–8%, the γ-globulin conjugates 0·5–7·5%, of the bonded uracil derivative. In the case of conjugates *Va* and *VIa*, and to a lesser extent also of compounds *Vb* and *VIb*, the increasing content of the bonded haptene was accompanied by a hypsochromic shift of the absorption maximum and increase in the extinction coefficient of the protein (at λ_{max} 280 nm). The absorption maximum of conjugates with a high content of the bonded haptene is shifted into the region, characteristic for low-molecular weight haptene. All the thus-prepared conjugates were uniform according to electrophoresis on agar gels. Our results show that the described synthetic method is more general and advantageous than other methods hitherto described for binding of compounds of the type *I* to proteins.

EXPERIMENTAL

Melting points were determined on a Boetius hot stage apparatus and are uncorrected. Unless stated otherwise, the solutions were evaporated on a vacuum rotatory evaporator at 40°C/15 Torr, and the compounds dried at 0·1 Torr over phosphorus pentoxide.

Compounds and Methods

Human serum albumin was prepared in the Forschungsinstitut für Impstoffe (Dessau, G.D.R.), bovine γ-globulin was purchased from Ferak Co. (West Berlin).

Paper chromatography was performed on a Whatman No 1 paper in the systems: S1, 2-propanol–conc. ammonia–water (7 : 1 : 2); S2, 1-butanol–acetic acid–water (5 : 2 : 3). Paper electrophoresis was carried out according to ref.[12] at 20 V/cm (1 h) in 0·1M triethylammonium hydrogen carbonate, pH 7·5 (E1). Gel electrophoresis was performed according to ref.[6]. Thin-layer chromatography on silica gel was carried out on Silufol UV_{254} plates (Kavalier, Votice, Czechoslovakia) in the systems: S3, chloroform–ethanol (9 : 1), S4, chloroform–ethanol (4 : 1). Compounds were detected by UV light (Chromatolight). The R_F and electrophoretic mobility values are given in Table III. Preparative gel chromatography was performed on a column (100 × 2 cm) of Sephadex G 50 coarse (Pharmazia, Sweden) in an ammonium carbonate buffer (10 g of ammonium carbonate in 1 l of water; pH adjusted to 8·7 with ammonia), elution rate about 1·2 ml/min, 3 ml fractions. The elution was followed by continuous UV absorption measurement (Uvicord). UV spectra were taken on a Specord UV-VIS spectrophotometer (Carl Zeiss, Jena, G.D.R.), quantitative determinations were done on a Spektromom 203 instrument (Hungarian Optical Works, Budapest, Hungary).

Determination of the Bonded Haptene Content in the Conjugate

a) Solutions of compound *V* and *VI* in ammonium carbonate, pH 8·7 (*vide infra*), were measured at the wavelength corresponding to absorption maximum of *IV* and after subtracting the absorption of a comparison protein solution of the same concentration, the content was calculated using molar extinction coefficient values for the corresponding compound *IV* (Table I).

b) Eluate containing the low molecular weight fraction after gel chromatography on Sephadex G 50 was measured at 400 nm (absorption of *p*-nitrophenoxide; the compounds *I* do not absorb

at this wavelength) and the *p*-nitrophenol concentration was calculated from its molecular extinction coefficient, $1{\cdot}8 \cdot 10^4$. The absorbance at the wavelength of the absorption maxima of compounds *IV* was also measured and the following molar extinction coefficients were used in the calculation (for *p*-nitrophenol: *Ia* 2 850 (266 nm), *Ib* 2 500 (271 nm), *Ic* 1 850 (280 nm), *Id* 1 525 (283 nm). The difference, calculated from the determination of *p*-nitrophenol and of the mentioned compounds, corresponds to the covalently bonded uracil derivative. The values, obtained by both these methods, are very similar.

p-Nitrophenyl Uracil-1-ylacetate (*IIa*)

A solution of the compound *Ia* (0·85 g; 5 mmol) and *p*-nitrophenol (0·70 g; 5 mmol) in dimethylformamide (30 ml) was cooled to 0°C and N,N′-dicyclohexylcarbodiimide (1·03 g; 5 mmol) was added. The mixture was set aside at 0°C for 1 h and at room temperature for 2 h, dioxane (50 ml) was added, the suspension filtered, the filtrate taken down *in vacuo* at 35°C and the residue codistilled (à 25 ml) with toluene (3×) and with dioxane. The residue was triturated with ether (20 ml) in order to induce crystallisation and the product was filtered and crystallized from ethanol, m.p. 214—218°C yield 70%. For $C_{12}H_9N_3O_6$ (291·2) calculated: 49·48% C, 3·11% H, 14·43% N; found: 49·42% C, 3·32% H, 14·31% N.

TABLE III
Chromatography and Electrophoresis

Compound	R_F				E_{Up} [a]
	S1	S2	S3	S4	
Ia	0·36	0·42	0·13	0·29	0·85
Ib	0·32	0·49	0	0·19	1·29
Ic	0·35	0·68	0·29	0·61	1·10
Id	0·38	0·66	0·30	0·50	0·90
IIa	—	—	0·56	0·79	—
IIb	—	—	0·65	0·75	—
IIc	—	—	0·65	0·84	—
IId	—	—	0·63	0·81	—
IIIa	0·38	0·48	0·05	0·11	0
IIIb	0·39	0·55	0·05	0·15	0·64
IIIc	0·44	0·55	0·14	0·30	0·51
IIId	0·47	0·57	0·16	0·34	0·44
IVa	0·48	0·77	0·10	0·34	0·54
IVb	0·42	0·81	0·13	0·47	0·87
IVc	0·50	0·82	0·28	0·65	0·81
IVd	0·51	0·81	0·28	0·72	0·72

[a] Electrophoretic mobility in E1 referred to uridine 2′(3)′-phosphate.

p-Nitrophenyl 5-Fluorouracil-1-ylacetate (*IIb*)

This compound was prepared from compound *Ib* (0·19 g; 1 mmol) and *p*-nitrophenol (0·14 g; 1 mmol) in dimethylformamide (2·5 ml) in the presence of N,N'-dicyclohexylcarbodiimide (0·21 g; 1 mmol) as described for the compound *IIa*. After the work-up procedure, the residue was triturated with light petroleum (3 ml), filtered and the product crystallised from ethanol, m.p. 199—201°C; yield 65%. For $C_{12}H_8FN_3O_6$ (309·2) calculated: 46·61% C, 2·51% H, 6·14% F, 13·59% N; found: 46·77% C, 2·68% H, 6·27% F, 13·81% N.

p-Nitrophenyl 5-Bromouracil-1-ylacetate (*IIc*)

Prepared from *Ic* (0·50 g; 2 mmol), *p*-nitrophenol (0·28 g; 2 mmol) and N,N'-dicyclohexylcarbodiimide (0·42 g; 2 mmol) in dimethylformamide (7 ml), similarly as described for *IIa*. The pure product *IIc* was obtained in an 80% yield by crystallisation from ethanol, m.p. 235 to 238°C. For $C_{12}H_8BrN_3O_6$ (370·2) calculated: 38·93% C, 2·18% H, 21·60% Br, 11·35% N; found: 38·61% C, 2·46% H, 21·98% Br, 11·12% N.

p-Nitrophenyl 5-Iodouracil-1-ylacetate (*IId*)

Prepared in 85% yield as described for the compound *IIc*, m.p. 213—219°C (ethanol). For $C_{12}H_8IN_3O_6$ (417·1) calculated: 34·55% C, 1·93% H, 30·42% I, 10·07% N; found: 34·68% C, 2·30% H, 30·83% I, 10·10% N.

Uracil-1-ylacetamide (*IIIa*)

Methanolic ammonia (30%; 3 ml) was added to a solution of the compound *IIa* (0·29 g; 1 mmol) in dimethylformamide (5 ml) and the mixture was set aside at room temperature overnight. After evaporation *in vacuo* and codistillation with toluene (4 × 10 ml), the residue was washed several times with ether and then crystallised from ethanol. Yield 75%, m.p. 284—289°C (sublimes above 230°C) (ref.[13]). For $C_6H_7N_3O_3$ (169·2) calculated: 42·60% C, 4·17% H, 24·84% N; found: 43·27% C, 4·40% H, 24·39% N. The following derivatives were prepared analogously: *IIIb* (from 0·5 mmol of *IIb*), yield 70%, m.p. 272—278°C (decomposition; 75% ethanol) (sublimes above 225°C). For $C_6H_6FN_3O_3$ (187·1) calculated: 38·50% C, 3·23% H, 10·16% F, 22·45% N; found: 38·66% C, 3·49% H, 10·41% F, 22·08% N. *IIIc* (from 0·5 mmol of *IIc*), yield 85%, m.p. 277—284°C (decomposition; 50% ethanol). For $C_6H_6BrN_3O_3$ (248·1) calculated: 29·05% C, 2·44% H, 32·21% Br, 16·94% N; found: 29·01% C, 2·90% H, 31·92% Br, 17·21% N. *IIId* (from 0·5 mmol of *IId*), yield 82%, m.p. 295—301°C (decomposition; water). For $C_6H_6IN_3O_3$ (295·0) calculated: 24·43% C, 2·05% H, 43·02% I, 14·25% N; found: 24·62% C, 1·87% H, 42·76% I, 14·40% N.

N-(5-Carboxypentyl)uracil-1-ylacetamide (*IVa*)

An aqueous solution of ε-aminocaproic acid (0·26 g; 2 mmol) and tetraethylammonium hydroxide (2 mmol) was evaporated *in vacuo*, the residue codistilled with dimethylformamide (3 × 5 ml) and dissolved in dimethylformamide (4 ml). After cooling to 0°C, a solution of compound *IIa* (0·29 g; 1 mmol) in dimethylformamide (4 ml) was added with stirring and the mixture was stirred for 3 h at room temperature. After addition of ether (25 ml) the mixture was decanted and the residue washed with ether (2 × 25 ml). The residue was dissolved in water (5 ml), acidified with hydrochloric acid and set aside for 2 h at 5°C. The product was collected on filter and washed

with small amount of water, m.p. 219—220°C; yield 70%. For $C_{12}H_{17}N_3O_5$ (283·3) calculated: 50·87% C, 6·05% H, 14·83% N; found: 51·32% C, 6·10% H, 14·44% N. The following derivatives were prepared analogously: *IVb* (from 0·5 mmol of *IIb*), yield 60%, m.p. 173—177°C. For $C_{12}H_{16}FN_3O_5$ (301·3) calculated: 47·83% C, 5·36% H, 6·31% F, 13·95% N; found: 48·05% C, 5·39% H, 6·18% F, 14·31% N. *IVc* (from 1 mmol of *IIc*), yield 65%, m.p. 198—202°C. For $C_{12}H_{16}BrN_3O_5$ (362·2) calculated: 39·79% C, 4·46% H, 22·07% Br, 11·61% N; found: 39·51% C, 4·80% H, 22·09% Br, 11·68% N. *IVd* (from 1 mmol of *IId*), yield 70%, m.p. 228—231°C. For $C_{12}H_{16}IN_3O_5$ (409·2) calculated: 35·22% C, 3·94% H, 31·02% I, 10·27% N; found: 35·61% C, 4·26% H, 30·59% I, 10·01% N.

Preparation of Conjugates *V* with Human Serum Albumin

To a solution of human serum albumin (69 mg; 1 μmol) in 0·2M borate buffer (pH 9·2; 2 ml) were added solutions of 10, 20, 40 or 80 μmol of the compound *II*, each in 0·5 ml of dimethyl sulfoxide. The mixture was set aside for 24 h at room temperature, applied (à 1 ml) to a column of Sephadex G 50 and eluted (*vide supra*). The high-molecular fractions (18—28) were combined, made up to the same volume and the content was determined spectrophotometrically. The low molecular weight portion (fractions 45—65) was worked up similarly and the content of compound *I* and *p*-nitrophenol was determined. The high molecular weight portion was then lyophilized. Yield and properties of thus-obtained compounds *V* are given in Table II.

Preparation of the Conjugates *VI* with Bovine γ-Globulin

To a solution of bovine γ-globulin (80 mg; 0·5 μmol) in a 0·2M borate buffer (pH 9·2; 2 ml) was added a solution of 5, 10, 20 or 40 μmol of the compound *II* in dimethyl sulfoxide (0·5 ml). The work-up procedure was the same as in the case of the conjugates *V*. Yields and properties of the products are given in Table II.

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Translated by M. Tichý.