NEW ADSORBENTS FOR THYMIDYLATE SYNTHASE AFFINITY CHROMATOGRAPHY

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Dedicated to the memory of Dr Karel Bláha.

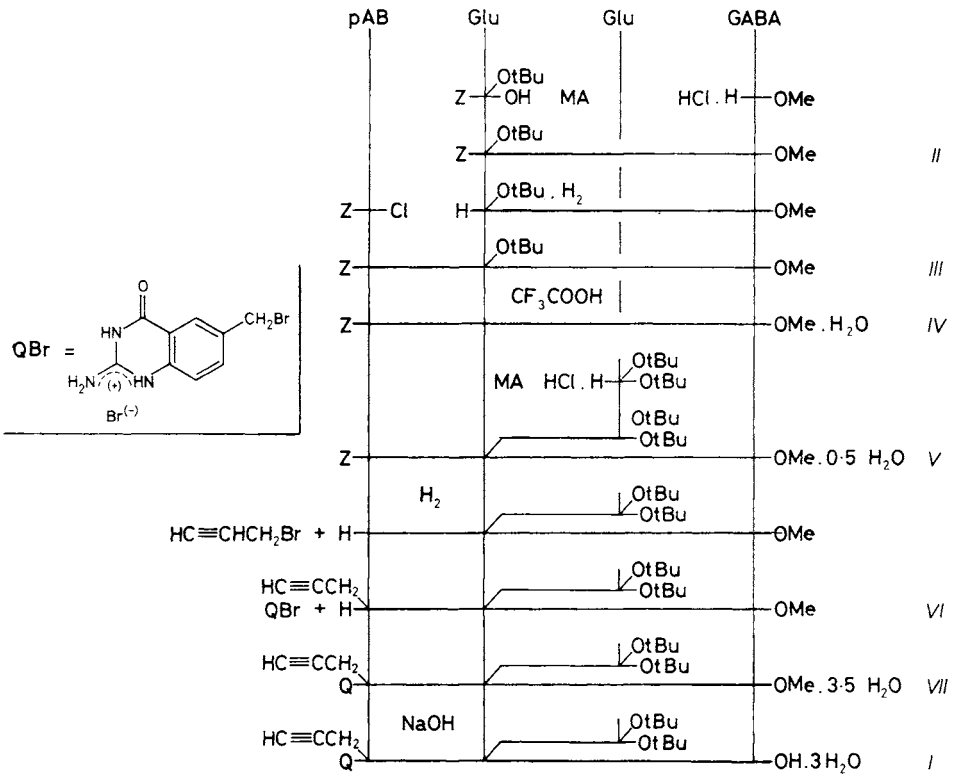
New affinity adsorbents, intended for chromatography of thymidylate synthase (EC 2.1.1.45) from different sources, consisting of *p*-[N-[(2-amino-4-hydroxy-6-quinazolinyl)-methyl]-N-2--propynylamino]benzoyl-γ-[α-(3-carboxypropylamino)]glutamyl-glutamyl immobilized either on macroporous copolymer of acrylonitrile and n-butyl acrylate or on macroporous polymer of acrylonitrile itself, both crosslinked with divinylbenzene and having aminoethyl groups, were obtained. Both adsorbents were found to be effective in dUMP-dependent binding of thymidylate synthase from regenerating rat liver.

Thymidylate synthase (EC 2.1.1.45) catalyzes reductive methylation of dUMP* with formation of dTMP and concomitant conversion of 5,10-methylenetetrahydrofolate to dihydrofolate[1]. As the only source of thymidylate de novo synthesis in a cell, the enzyme is an actual target for anticancer, antiviral and antifungous chemotherapy[2,3] and a possible target for antiparasitic chemotherapy[4]. Hence properties of thymidylate synthase of different origin are of great interest.

Purification of animal thymidylate synthase is a difficult task because of its lability and low levels in tissues. The enzyme has been purified most successfully by affinity chromatography on a Sepharose-bound analogue of 5,10-methylenetetrahydrofolate[1,5]. The dUMP-dependence of thymidylate synthase binding to such an adsorbent enables specific both adsorption and elution of the enzyme[6]. Starting comparative studies of thymidylate synthases from regenerating rat liver and the type-

* The amino acids used in this paper are of L configurations. Nomenclature and symbols follow the published recommendations (Eur. J. Biochem. *138*, 9 (1984); *15*, 203 (1970)). Additional abbreviations: pAB, 4-aminobenzoic acid; GABA, 4-aminobutanoic acid; DMF, N,N-dimethylformamide; THF, tetrahydrofuran; MA, mixed anhydride.

worm, *Hymenolepis diminuta*, we found the enzyme from both sources to bind very poorly to 10-formyl-5,8-dideazafolate-aminoethyl-Sepharose, proved earlier to be effective by purification of thymidylate synthase of viral, bacterial and mammalian orgin[1,5,7]. In order to create a more universal affinity adsorbent, we applied as an affinant 10-propargyl-5,8-dideazapteroyl-γ-glutamyl-glutamic acid[8,9], reported to belong to the strongest inhibitors of thymidylate synthase[10,11]. This compound immobilized on aminoethyl-Sepharose, by means of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, expresses dUMP-dependent binding of both the typeworm and regenerating rat liver thymidylate synthases (unpublished). However, the structure of the above-mentioned adsorbent was equivocal, since three carboxyl groups of the ligand were available for coupling. Besides, Sepharose-based columns have limited mechanical stability and only moderate flow properties. Therefore we have synthesized proligand *I* (Scheme 1) to ensure (i) an unequivocal binding between the ligand and its support and (ii) separation of the ligand from the support with the



SCHEME 1

GABA spacer, and have anchored the proligand on macroporous acrylic acid derivatives-based polymers.

Scheme 1 shows the outline of the synthesis of proligand *I*. All steps but the synthesis of compound *VI* and *VII* proceeded unequivocally. Compounds *VI* and *VII* have been purified by chromatography on silica gel. Different amounts of the proligand *I* were coupled with each of two macroporous polymers: (i) a copolymer of acrylonitrile and n-butyl acrylate (1 : 1) crosslinked with divinylbenzene (10%) and chemically modified with ethylenediamine[12] (polymer A) and (ii) a polymer of acrylonitrile itself crosslinked with divinylbenzene (10%) and chemically modified with lithium aluminium hydride[13] (polymer B). The polymers were selected for their chemical, physicochemical, mechanical and flow properties. Their characteristic data are listed in Table I. Coupling of the proligand *I* with polymers was performed using the mixed anhydride with isobutyl chlorocarbonate and completness of the anchoring checked by UV measurements and TLC. Trifluoroacetic acid served for removal of t-butyl groups from the proligand bound to a polymer.

Adsorbents of both types displayed dUMP-dependent binding thymidylate synthase from regenerating rat liver as well as expected excellent flow properties. Results of affinity chromatography of the enzyme employing these adsorbents will be published elsewhere.

EXPERIMENTAL

Chemicals. Z-pAB-Cl[14], Z-Glu(OtBu)[15,16] HCl.GABA-OMe[17] and HCl.Glu(OtBu)$_2$[18] were prepared by previously described procedures. Propargyl bromide was used as an 80% w/w solution in toluene (Aldrich 2-Amino-6-bromo-4-hydroxyquinazoline hydrobromide and polymers were generous gifts from Dr Terence R. Jones from the Institute of Cancer Research, Sutton, U.K. and Prof. Bożena N. Kolarz from the Institute of Organic and Polymer Technology, Technical University, Wrocław, Poland, respectively. CF_3COOH containing maximum 0·1%

TABLE I
Characteristic data of used polymers

Characteristics		Polymer A[a]	Polymer B
Amino groups	— mmol g^{-1}	1·5	0·2
Porosity	— %	10	65
Weight swelling ratio	— g H_2O/g	2·60	1·97
Specific surface area	— m^2 g^{-1}	0·30	88
Bead diameter	— mm	0·08—0·12	0·08—0·12

[a] For chemical composition see text.

water, 10% Pd on active carbon and N,N-dimethylacetamide (DMA) were from Merck, Fluka and Aldrich, respectively. Tertiary amines were distilled over ninhydrin, ethers over sodium, and chloroform over a mixture of anhydrous K_2CO_3 and P_2O_5. DMF was azeotropically distilled with water and benzene in vacuo. Hexane and benzene were refined with conc. H_2SO_4 and distilled over sodium. Other solvents and isobutyl chlorocarbonate were distilled. Ethers, methanol and remaining solvents were stored over sodium wire, activated (250°C) molecular sieves 3 Å and 4 Å, respectively.

Experimental arrangement and procedures. General. Reactions were monitored and the homogeneity of products was checked on silica gel plates (DC Alufolien Kieselgel 60 Merck) in the following solvent systems: chloroform–methanol–acetic acid (38 : 2 : 1) (S1), chloroform–methanol–conc. ammonia (6 : 5 : 1) (S2), chloroform–methanol (5 : 1) (S3) and benzene–pyridine–acetic acid (20 : 2 : 1) (S4). Spots were visualized with chlorine–tolidine reagent and with ninhydrin. Organic solutions were dried with anhydrous Na_2SO_4 and solvents were removed in vacuo on rotatory evaporator at a bath temperature not exceeding 30°C. For gravity chromatography, Merck silica 60 and UV detector at 254 nm (KB-5301 Zopan, Poland) were used. Melting temperatures were determined on a Boëtius apparatus and are uncorrected. Samples for elemental analysis were dried over P_2O_5 in vacuo ($p < 130$ Pa) at 65°C for 3 h and analysis performed on a Perkin–Elmer analyser. Specific rotations were measured with a polarimeter Polamat A (Zeiss, G.D.R.). Mass spectra were taken with double-focusing mass spectrometer Varian MAT 711, emitter current 19—20 mA, acceleration voltage (8 + 3) kV. UV measurements were done with a Specord UV-VIS (Zeiss, G.D.R.).

Methyl Benzyloxycarbonyl (γ-t-butyloxy)glutamyl-γ-aminobutyrate (*II*)

A magnetically stirred solution of Z-Glu(OtBu) (10·12 g; 30 mmol) and N-methylmorpholine (3·33 ml; 30 mmol) in THF (60 ml) was cooled to −20°C and isobutyl chlorocarbonate (4·13 ml; 30 mmol) added dropwise. In 4 min, HCl.GABA-OMe) 4·61 g; 30 mmol) and N-methylmorpholine (3·33 ml; 30 mmol) were introduced. The stirring was continued for 15 min at −10°C, 15 min at 0°C and then 15 min at 10°C. THF was evaporated, the residue dissolved in ethyl acetate and washed with a mixture of saturated $NaHCO_3$ and water (1 : 1), 0·5M-H_2SO_4, a mixture of brine and water (1 : 1) and brine. Evaporation of ethyl acetate left a colourless oil (13·5 g; 102%). After 3 days at −10°C, the oil went glass (13·1 g) which was crystallized from diethyl ether by the addition of hexane. The yield was 10·5 g (80%) of *II*; m.p. 54·5—55·5°C, $[\alpha]_D^{22.5}$ −45·2° (*c* 1·0; methanol); R_F 0·71 (S1).

Methyl *p*-Benzyloxycarbonylaminobenzoyl-(γ-t-butyloxy)glutamyl-γ-aminobutyrate (*III*)

Through a magnetically stirred solution of crude *II* (13·1 g; 30 mmol) in methanol (150 ml) containing 10% Pd on active carbon (2·6 g) at 21°C hydrogen was bubbled for 2·5 h (to cessation of carbon dioxide evolution against $Ba(OH)_2$). The catalyst was filtered and methanol evaporated to leave 9·0 g of 90% amine (90% yield). To the amine (9·0 g; 27 mmol), dissolved in dioxane (27 ml), Z-pAB-Cl (7·82 g; 27 mmol) was added and then tributylamine introduced dropwise. After 20 min, dioxane was evaporated, the residue taken up in ethyl acetate and washed with 0·05M-H_2SO_4 and 1M-$NaHCO_3$. Ethyl acetate was evaporated, to the residue diethyl ether (25 ml) added and evaporated. The same was repeated once more with ether and once with hexane (25 ml each) giving 15·0 g (100% yield) of white crude *III*. Crystallization from ethyl acetate–hexane afforded 9·88 g (66% yield) of *III*; m.p. 129—132°C, $[\alpha]_D^{20}$ + 6·5° (*c* 1·0; methanol); R_F 0·47 (S1), 0·80 (S3), 0·46 (S4). For $C_{29}H_{37}N_3O_8$ (555·6) calculated: 62·69% C, 6·71% H, 7·56% N; found: 62·55% C, 6·74% H, 7·48% N. Mass spectrum *m/z*: 555 (M^+).

Methyl *p*-Benzyloxycarbonylaminobenzoyl-glutamyl-γ-aminobutyrate Monohydrate (*IV*)

Compound *III* (2·778 g; 5 mmol) was dissolved in CF_3COOH which after 15 min was evaporated. To the residue, diethyl ether (50 ml) was added and evaporated. That was repeated twice more to give 2·657 g of a white solid which was washed with diethyl ether (2 × 50 ml, 2 × 10 ml), ethyl acetate (12 ml) and diethyl ether (12 ml). The yield was 2·468 g (99%) of crude *IV*. Crystallization from ethanol afforded 2·024 g (78%) of *IV*; m.p. 190–192°C, $[\alpha]_D^{20}$ +11·8° (*c* 1·0; methanol); R_F 0·18 (S1), 0·80 (S2), 0·39 (S3). For $C_{25}H_{29}N_3O_8.H_2O$ (517·5) calculated: 58·02% C, 6·04% H, 8·12% N; found: 58·25% C, 6·10% H, 8·17% N. Mass spectrum *m/z*: 499 (M^+).

Di(t-butyl) *p*-Benzyloxycabonylaminobenzoyl-γ-[α-(3-methoxy-carbonylpropylamino)]glutamyl-glutamate Hemihydrate (*V*)

A magnetically stirred solution of *IV* (2·00 g; 3·86 mmol) and N-methylmorpholine (0·45 ml; 4·0 mmol) in DMF (16 ml) was cooled to −15°C and isobutyl chlorocarbonate (0·55 ml; 4·0 mmol) added. After 4 min, $HCl.Glu(OtBu)_2$ (1·247 g; 4·2 mmol) and N-methylmorpholine (0·47 ml; 4·2 mmol) were introduced. The stirring was continued for 15 min at −10°C, 15 min at 0°C and then 15 min at 10°C. DMF was evaporated, the residue dissolved in ethyl acetate and washed with 0·5M-H_2SO_4, a mixture of saturated $NaHCO_3$, brine and water (1 : 1 : 1) and a mixture of brine and water (1 : 1). Ethyl acetate was evaporated to give 2·934 g of a cream-coloured solid which was crystallized from ethyl acetate by addition of hexane. The yield was 2·67 g (92%) of *V*; m.p. 136–140°C, $[\alpha]_D^{20}$ −9·0° (*c* 1·0; methanol); R_F 0·45 (S1), 0·89 (S2), 0·79 (S3), 0·34 (S4). For $C_{38}H_{52}N_4O_{11}.0{\cdot}5\ H_2O$ (749·9) calculated 60·86% C, 7·12% H, 7·48% N; found: 60·86% C, 7·16% H, 7·45% N. Mass spectrum *m/z*: 741 (M^+).

Di(t-butyl) *p*-N-2-Propynylaminobenzoyl-γ-[α-(3-methoxy-carbonylpropylamino)]glutamyl-glutamate (*VI*)

Through a magnetically stirred solution of *V* (2·25 g; 3·0 mmol) in methanol (30 ml), containing 10% Pd on active carbon (0·45 g), at 21°C, hydrogen was bubbled for 20 min (to cessation of carbon dioxide evolution against $Ba(OH)_2$). The catalyst was filtered and methanol evaporated to afford 1·84 g (100% yield) of amorphous solid; R_F 0·28 (S1), 0·84 (S2), 0·73 (S3). To a magnetically stirred solution of the amine in DMA (3 ml), $CaCO_3$ (0·3 g; 3 mmol) and propargyl bromide (0·33 ml; 3 mmol) were added. The stirring was continued for 165 h at 21–22°C, DMA evaporated, the residue dissolved in chloroform–methanol (19 : 1) system (2·5 ml), applied to a column (150 × 3·2 cm, 700 g silica gel) and eluted with the same system. Fractions containing only *VI* were collected and evaporated. The residue was dried over P_2O_5 in vacuo at $p < 130$ Pa to give 0·72 g (37%) of amorphous *VI*; R_F 0·46 (S1), 0·85 (S2), 0·79 (S3), 0·29 (S4).

Di(t-butyl) *p*-[N-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]--N-2-propynylamino]benzoyl-γ-[α-(3-methoxycarbonylpropylamino)]-glutamyl-glutamate (*VII*)

To a magnetically stirred solution of *VI* (0·645 g; 1 mmol) in DMA (2·5 ml), 2-amino-6-bromomethyl-4-hydroxyquinazoline hydrobromide (0·335 g; 1 mmol) and $CaCO_3$ (0·1 g; 1 mmol) were added. The stirring was continued for 168 h at 25–27°C, DMA evaporated, and the residue taken up in chloroform. Inorganic salts were filtered and chloroform evaporated to leave 1·00 g of red solid which was dissolved in chloroform and rapidly precipitated with diethyl ether (40 ml). The obtained fleshcoloured solid (0·84 g; 104%), dissolved in chloroform–methanol (97 : 3)

system (5 ml), was applied to a column (39 × 3·5 cm, 210 g silica gel) and eluted with chloroform-methanol (19 : 1) (500 ml) and chloroform–methanol (10 : 1). Fractions containing only *VII* were collected, evaporated and dried to afford 450 mg (55%) of amorphous *VII*; $[\alpha]_D^{20}$ +4·3 (*c* 1·0; DMF); R_F 0·10 (S1), 0·82 (S2), 0·54 (S3). For $C_{42}H_{55}N_7O_{10}.3{\cdot}5\ H_2O$ (881·0) calculated: 57·25% C, 7·09% H, 11·14% N; found: 57·35% C, 6·52% H, 10·83% N. Mass spectrum *m/z*: 819 $(M)^+$.

Di(t-butyl) *p*-[N-[(2-Amino-4-hydroxy-6-quinazolinyl)methyl]-
-N-2-propynylamino]benzoyl-γ-[α-(3-carboxypropylamino)]glutamyl-glutamate (*I*)

To *VII* (41 mg; 50 μmol), suspended in dioxane (300 μl), 1M-NaOH (100 μl; 100 μmol) and 0·1M-NaOH (100 μl; 10 μmol) were added (pH about 13·5). The reaction mixture was stirred for 45 min at 25°C, whereby, after 10 min, some of a new solid precipitated. Dioxane (700 μl) and water (900 μl) were added (pH about 11·5) and the whole, stirred, was acidified with 0·1M-HCl (1·1 ml; 110 μmol). The resulting precipitate was filtered and washed with water (4 × 2 ml) and diethyl ether (2 ml) giving 34 mg (84%) of *I*; m.p. 183—187°C (dec.); R_F 0·65 (S2), 0·20 (S3). For $C_{41}H_{53}N_7O_{10}.3\ H_2O$ (858·0) calculated: 57·39% C, 6·93% H, 11·44% N; found: 57·66% C, 6·45% H, 10·90% N. Mass spectrum *m/z*: 805 (M^+).

Coupling the Proligand *I* to a Polymer

A stirred solution of proligand *I* (8 mg; 10 μmol) in DMF (100 μl) and THF (10 μl) was cooled to −15°C and 1M solution of N-methylmorpholine in THF (10 μl; 10 μmol) and 1M solution of isobutyl chlorocarbonate in THF (10 μl; 10 μmol) were added. The stirring was continued for 7 min at −15°C. Then the whole was added to a suspension of sediment polymer A or B in THF (10 ml) and left for 2 h at −5°C with occasional stirring. After this period of time, UV adsorption measurement displayed 1·2% of proligand uncoupled to polymer. In this procedures, different ratios of proligand *I* to a polymer could be used.

Removal of t-Butyl Groups from the Proligand *I* Coupled to a Polymer

Polymeric samples (1 ml) with bound proligand *I* were treated successively at 15 min intervals with the following mixtures of THF and CH_2Cl_2: 2 : 1, 1 : 1, 1 : 2, 0 : 2 (2 ml each) and next with trifluoroacetic acid in CH_2Cl_2: 5%, 10% and 20% (2 ml each). Then the samples were left for 3 h in 30% trifluoroacetic acid in CH_2Cl_2 with occasional stirring, washed with THF (10 ml), 30% solution of triethylamine in THF (10 ml) and with gradient of THF and water, from 0 to 100% water, to pH 7·0.

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