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Synthesis of gem-bisphosphonic methotrexate conjugates and their biological response towards Walker's osteosarcoma

G Sturtz[1], H Couthon[1], O Fabulet[1], M Mian[2], S Rosini[2]

[1]*Laboratoire de Chimie Hétéro-Organique, UFR Sciences et Techniques de Brest, BP 452, 29275 Brest Cedex, France*
[2]*Istituto Gentili, Via Mazzini, 112, 56100 Pisa, Italy*

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Introduction

We have recently shown that methotrexate gem-bisphosphonic prodrugs (MTX; fig 1) have an interesting therapeutic action on human osteosarcomas implanted in nude mice [1]; and it has previously been demonstrated that compound **9b** used at 5 to 6-fold lower molar doses already has a better activity than MTX itself.

These results seemed to confirm the hypothesis of drug targeting with the aid of bisphosphonates. With this mind, it appeared of interest to carry out a pharmacological study on the amino-gem-bisphophonate.

In this study a new convergent synthesis has been presented of compound **9b** (fig 2), which is more rapid than initially described [1]; and we have prepared a new gem-bisphophonate prodrug **10b** in the same way (fig 3).

Once the 2 methotrexate prodrugs **9b**, **10b** were obtained, they were tested on Walker sarcoma models implanted in the rat dorsoscapular region.

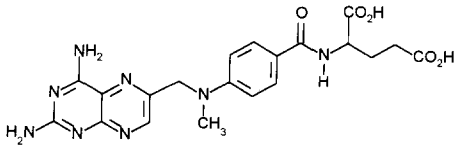
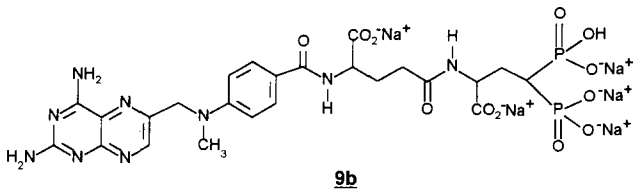
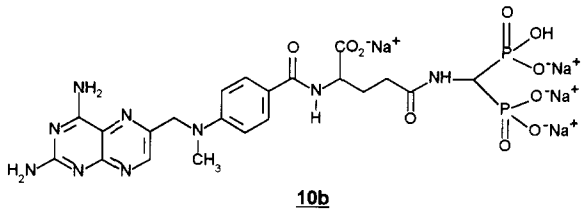


Fig 1. Methotrexate.



9b

Fig 2. Compound **9b**.



10b

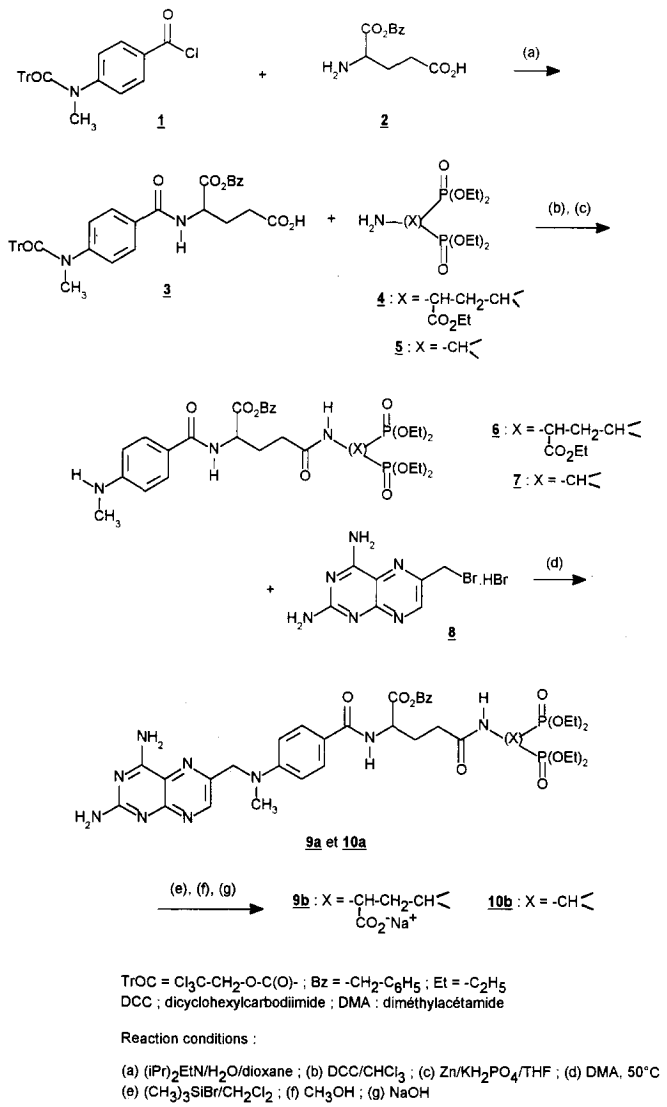
Fig 3. Compound **10b**.

Chemistry

The preparation of compound **9b** which has been previously described [1], involved an initial coupling between the glutamic acid **2** α-benzylester and the gem-bisphophonate derivative **4** before condensation of the chloride of *N*-protected *p*-(*N*-methylamino)-benzoic acid **1**. This synthesis required the protection of the amine function.

An attempt was made to reduce the number of reaction steps by modifying the coupling order of different fragments of the molecule.

To achieve this, the chloride of the *N*-protected *p*-(*N*-methylamino)benzoic acid **1** was condensed with glutamic acid, the carboxylic acid function in the α position being protected in a benzylic ester form **2** [2]



TrOC = Cl_3C-CH_2-O-C(O)- ; Bz = -CH_2-C_6H_5 ; Et = -C_2H_5
DCC ; dicyclohexylcarbodiimide ; DMA : diméthylacétamide

Reaction conditions :

(a) $(iPr)_2EtN/H_2O$/dioxane ; (b) DCC/$CHCl_3$; (c) Zn/KH_2PO_4/THF ; (d) DMA, 50°C
(e) $(CH_3)_3SiBr/CH_2Cl_2$; (f) CH_3OH ; (g) NaOH

Scheme 1.

in the presence of diisopropylethylamine in a mixture of dioxane/water (3:4) according to the protocol described by Piper [3]. This procedure yielded compound **3**.

The acid **3** was then coupled to the gem-bisphophonic derivative **4** or **5** with the aid of dicyclohexylcarbodiimide (DCC) [4]. The reaction was carried out in chloroform at room temperature; the compounds were obtained in good yield from the condensate. The preparation of the amino-gem-bisphophonic derivatives **4** and **5** was carried out according to [5] and [6].

The amines **6** and **7** were then deprotected *via* the zinc powder in tetrahydrofuran in the presence of potassium hydrogen phosphate (pH 4–5) [7].

The final coupling was carried out by a nucleophilic substitution between the amine **6** and **7** and the 2,4-diamino-6-bromomethylpteridine **8** according to the procedure described by Piper [8]. The reaction was carried out in dimethylacetamide (DMA) at 50°C, without it being necessary either to protect the amine functions of the pteridine ring, or to trap the bromohydric acid formed by an auxiliary base [8].

Hydrolysis of the phosphonic esters took place *via* trimethylsilylbromide [9] followed by methanolysis. Finally, the 2 sodium salts **9b** and **10b** were isolated after saponification of the ethyl and benzyl esters.

Conclusion

This new synthetic approach to obtaining compound **9b** proved to be much more efficient, as the overall yield passed from 11% [1] to 43%.

This same convergent strategy allowed the new prodrug **10b** to be obtained by starting with the tetraethylester of 1-aminomethylenebisphosphonic acid **5** [6].

Pharmacology

Two types of experiments carried out on the Walker sarcoma models (from the M Negri Institute) allowed a comparison to be made of the biological activity of the 2 bisphosphonate carriers, 2-amino-4,4-bis(phosphonato)butanoic acid (**BP4**) and 1-aminomethylenebisphosphonic acid (**BP5**) (fig 4), and our 2 prodrugs **9b** and **10b** with that of the bisphosphonate reference compound (4-amino-1-hydroxy)butyl-1,1-bisphosphonate (**AHBuBP**) (fig 4).

Experiment 1

Sprague–Dawley (SD) rats weighing 150–200 g were injected with 5 x 10^6 Walker sarcoma cells [10]. At the same time, the animals were treated with the bisphosphonic derivatives in the following manner.

Table I. Bisphosphonate compounds used to treat 2 animal models of Walker's osteosarcoma.

Compound	*Mol weight (g/mol)*	*Weight (mg/kg/sc)*	*mmol*
AHBuBP	249	2.5	0.010
9b	809	10	0.012
BP4	263	4	0.015
10b	715	8.8	0.012
BP5	191	2.4	0.012

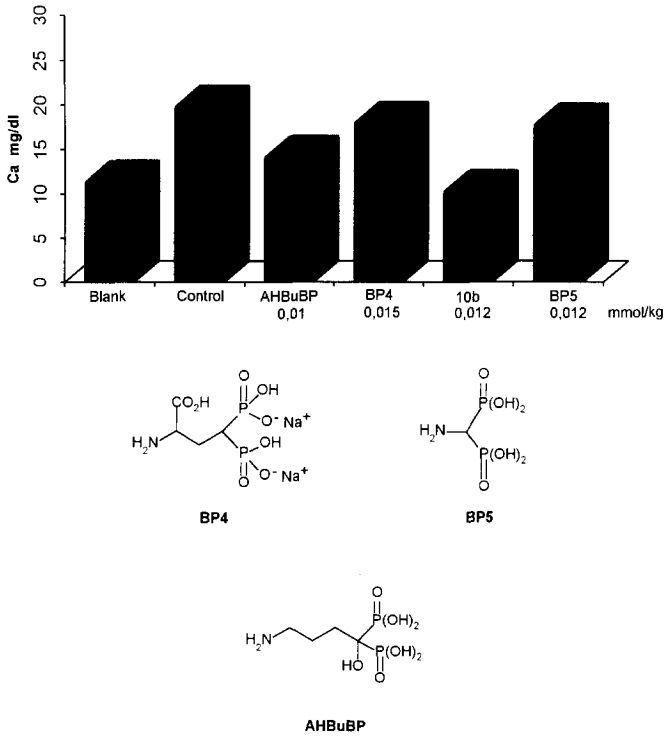


Fig 4. Effect of some BPs on the plasma calcium concentration in the rat, induced by W 256/B.

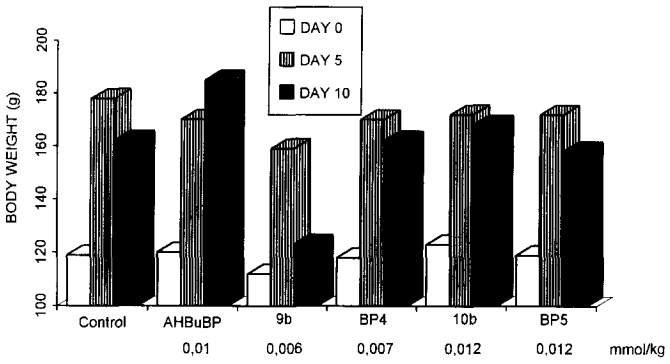


Fig 5. Evolution of body weight during treatment.

The influence of these bisphosphonates on the calcium concentration in the plasma was measured (fig 4). The increase in calcium reflected the evolution of metastatic concentration.

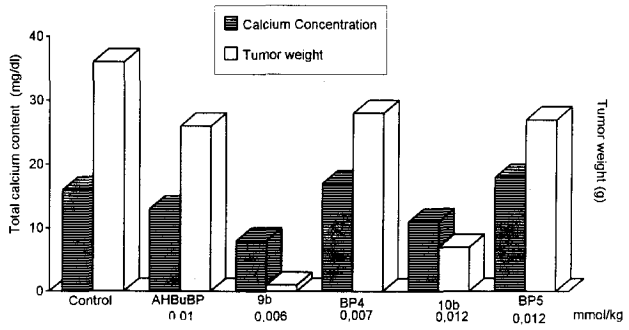


Fig 6. Effect of some BPs on the plasma calcium concentration and tumor weight after 10 d treatment.

Results

The administration of prodrug **9b** was interrupted after 8 d at a dose of 0.012 mmol/kg/d; the treated animals died without developing tumors. Due to this, we were not able to carry out measurement of plasma calcium concentrations for the studied molecule.

However, it can be seen from figure 4 that the vector **AHBuBP** and the bisphosphonate carriers **BP4** and **BP5** show a decrease in plasma calcium concentration. When compared to the control, with a value of 26.8%, 7.1% and 8.1% respectively, the prodrug **10b** completely inhibited the increase in calcium level in the plasma. Also, rats treated with **10b** did not develop tumors and were in good physical condition.

Experiment 2

The experimental model consisted of the sc injection of tumor fragments in the rat dorsoscapular region. The administration of drugs was carried out 48 h after tumor induction and lasted 10 d. The evolution in body weight of the animals, tumor growth and calcium concentrations were measured throughout the treatment period (figs 5, 6).

Results

It should be noted that the dose of the prodrug **9b** was divided into 2 aliquots (0.006 mmol/kg) in an attempt to reduce the toxicity encountered in the first experiment; fig 5 shows the variations in weight of the animals during the treatment period. This measurement allows us to obtain a certain evaluation of the toxicity of the compound. The histograms demonstrate that even at a dose of 0.006 mmol/kg,

compound **9b** is still toxic (significant loss of weight), whereas with the other 4 compounds and notably **10b** the animals seemed to maintain a good physical condition. Figure 6 shows some interesting results: the histograms of plasma calcium concentrations indicate that the lowest value is found in prodrug **9b**. Therefore tumor development is practically totally inhibited by this compound. However, it should be noted that the rats treated with these compounds are in poor physical condition: their coats are rough (or with spiky hair) and their stools are soft.

With the prodrug **10b** tumor development was controlled and the rats were found to be in good physical condition. On the other hand, the simple bisphosphonate, *ie* the reference drug **AHBuBP**, and the 2 carriers **BP4** and **BP5** only slightly inhibited tumor growth.

This clear difference between the vector and the prodrug unquestionably valorizes the present system of vectorization.

Conclusion

From these preliminary results it appears that in the 2 Walker osteosarcoma models studied, the 2 prodrugs methotrexate **9b** and **10b** showed interesting results regarding the control of bone tumor development, as **9b** at a dose of 0.006 mmol/kg/d totally inhibited tumor growth and **10b** at a dose of 0.012 mmol/kg/d was able to control tumor growth.

Due to the present state of these biological tests, it is difficult to explain the relative variation in toxicity between these 2 prodrugs whose bisphosphonate carrier structures, **BP4** and **BP5**, vary only slightly.

It therefore appears necessary to conduct further studies and to investigate pharmacomodulation at the carrier level so as to identify the compound likely to provide the best therapeutic index.

Experimental protocols

Chemistry

The melting points were determined on a Köfler apparatus. The melting points of the compounds containing a pteridine nucleus could not be measured. Decomposition of these molecules was observed > 180°C.

^{1}H- and ^{13}C-NMR spectra were recorded on a Brucker AC 300 (300 MHz) spectrometer with 3-trimethylsilylperdeuteropropanoic acid sodium salt (TMPS) as reference for D_2O and tetramethylsilane (TMS) for the organic solvents. The ^{31}P-NMR spectra were recorded on a Jeol JNM-FX 100 FT machine with phosphoric acid (H_3PO_4) memory point as an 85% solution in water.

Analytical thin-layer chromatography (TLC) was performed on silica-gel sheets (Merck, DC-Alufolien, Kieselgel, Germany; 60 F_{254}), which were scanned under ultraviolet light (ν = 254 nm). Preparative chomatography were carried out on silica-gel columns, Merck 60 (70–230 mesh). The compounds were prepared according to the procedures described: **1** [11]; **2** [4]; **4** [1]; **5** [5]; **8** [7].

*4[N,N-((2,2,2-Trichloroethoxycarbonyl)methyl)amino]benzoyl-glutamic α-benzyl ester **3***

To a 7 g (0.03 mol) solution of the α-benzyl ester of glutamic acid **2** and 10.4 ml (0.06 mol) diisopropylethylamine in 200 ml water/dioxane (4:3), cooled in 0–5°C, was added dropwise 11.5 g (0.03 mol) chloride of acid **1** diluted in 40 ml dioxane. After 30 min agitation, the water/dioxane mixture was evaporated and the resulting compound recovered in water. After passing through an acid solution by using a solution of potassium hydrogen sulfate, acid **3** was extracted with chloroform, and the solvent was then evaporated after drying on magnesium sulfate to give 13.8 g of white powder (yield: 85%; fusion point, 135–136°C).

^{1}H-NMR ($CDCl_3$) δ: 2.5 (m, 4H, CH_2-CH_2), 3.5 (s, 3H, CH_3), 4.9 (m, 3H, CH_2 and CH), 5.3 (s, 2H, CH_2-C_6H_5), 7.5 (s, 5H, C_6H_5), 7.8 (AB, 4H, H arom), 9.2 (s large, 1H, H acid).

*Nα-[4-(N-Methylamino)benzoyl]-Nδ-[1-ethoxycarbonyl-3,3-bis(diethylphosphono)propyl]glutamine benzyl ester **6***

1.7 g (8.25 mmol) dicyclohexylcarbodiimide were added dropwise to a mixture of 4.25 g (8.25 mmol) acid **3** and 3.3 g (8.25 mmol) amine **4** in 100 ml chloroform. After 12 h agitation at room temperature, the dicyclohexylurea was filtered through Celite and the chloroform evaporated. After acid-base extraction, the residue was purified by chromatography on a silica-gel column (mobile phase ethyl acetate/ethanol, 30:1) to give 7.2 g of the N-protected compound (93%) **6**.

A solution of 1.3 g of KH_2PO_4 in 13 ml H_2O was poured over the preceding 7.2 g, dissolved in 100 ml THF. Zinc powder (8 g), previously activated in 5% HCl solution, was added dropwise. Vigorous agitation was maintained for ≈ 24 h. After filtration and evaporation of THF, the residue was recovered in a water/chloroform mixture (4:5). Following concentration of the organic phase, 6 g of the amine **6** were obtained as a semi-solid (98%, ^{31}P-NMR ($CDCl_3$): 23.4 (d, $J^2_{P\text{-}P}$ = 10 Hz) and 23.2 (d, $J^2_{P\text{-}P}$ = 10 Hz)).

*Nα-[4-(N-Methylamino)benzyl]-Nδ-[1,1-bis(diethylphosphono)-methyl]glutamine benzyl ester **7***

The experimental protocol was the same as that used to obtain compound **6**. Starting with 2.5 g of amine **4** (8.25 mmol), 5 g of an intermediate compound were obtained after purification on a silica-gel column (mobile phase, ethylacetate/ethanol: 20:1).

The free amine **7** was then quantitatively obtained as a yellow oil by the action of zinc powder (^{31}P-NMR: 16.3 (s)).

*Nα-[4-amino-4-deoxy-N-10-methylpteroyl]-Nδ-[1-ethoxycarbonyl-3,3-bis(diethylphosphono)propyl]glutamine benzyl ester **9a***

1.5 g (4 mmol) pteridine bromohydrate **8** were dissolved in 15 ml dimethylacetamide at 50°C. To this, 3.8 g (0.005 mol) amine **6**, diluted in 5 ml DMA, were added dropwise. After 4 h agitation at 50°C, the solvent was evaporated and the residue recovered in 80 ml $CHCl_3$ and then washed in water. After

concentration of the chloroformic phase, the residue was purified on a silica-gel column (mobile phase, chloroform/ethanol: 10:1), followed by dissolution in 5 ml $CHCl_3$ and precipitation in 50 ml ether, to give 2.7 g of a fine yellow powder (80%, ^{31}P-NMR: 23.31 (s) and 23.34 (s)).

Nα-[4-amino-4-deoxy-N-10-methylpteroyl]-Nδ-[1,1-bis-(diethylphosphono)methyl]glutamine benzyl ester ***10a***

The condensation of amine **7** with 2,4-diamino-6-bromomethylpteridine **8** was carried out according to the preceding protocol. Starting with 3.3 g (0.005 mol) amine **7**, 1.7 g of compound **10a** were obtained after purification on a silica-gel column (mobile phase, chloroform/ethanol: 8:1), in the form of a bright yellow powder (62%, ^{31}P-NMR: 16.5 (s)).

Sodium quadrisalt of Nα-[4-amino-4-deoxy-N-10-methylpteoryl]-Nδ-[1-carboxylato-3,3-bis(phosphonato)propyl]glutamine ***9b***

Hydrolysis of phosphonic esters. 5.7 ml (43 mmol) trimethylsilylbromide were added to a solution of 5.0 g (5.4 mmol) ester **9** in 80 ml methylene chloride. After agitation at room temperature for 48 h, the methylene chloride was evaporated. The solid residue was recovered in 140 ml methanol and agitated at room temperature for 45 min. Following evaporation of methanol, 5.3 g bisphosphonic acid were quantitatively obtained in the form of a yellow powder. The potentiometric measurement of bromohydrate ions by silver nitrate indicated the presence of 2 bromohydrates.

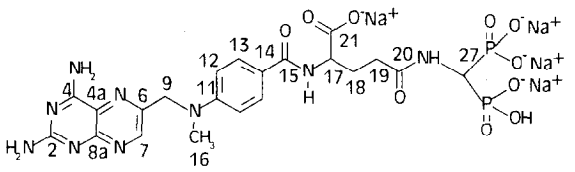
Saponification of carboxylic esters. 20 ml methanol was added to 5.3 g (5.4 mmol) bisphosphonic acid dibromhydrate **9a**, and the mixture refluxed. 37.8 ml (37.8 mmol) of NaOH solution (1 N) was then added dropwise and the reaction mixture agitated at 75°C for 8 h. Once the solution was concentrated, the salt was precipitated with methanol to give 4.2 g sodium pentasalt **9b** as a yellow powder (97%, NMR: in agreement with [1]).

Sodium quadrisalt of Nα-[4-amino-4-deoxy-N-10-methylpteroyl]-Nδ-[1,1-bis(phosphonato)methyl]glutamine ***10b***

Phosphonic ester hydrolysis. 3.2 ml (23 mmol) trimethylsilylbromide were used with 4 g (40 mmol) ester **10** according to the protocol described previously. 2.8 g were obtained as an ochre powder after dissolution in 5 ml methanol and precipitation in 50 ml ether.

Carboxylic ester saponification. 19.1 ml NaOH (1 N) were added dropwise to 2.8 g (30 mmol) bisphosphonic acid **10a** in 15 ml refluxed methanol. After 4 h at 75°C, the sodium quadrisalt was precipitated by supplementary addition of methanol, yielding 1.8 g golden yellow powder (85%, NMR: table II).

Table II. NMR of compound **10b**.



^{1}H-NMR (D_2O)	*^{13}C-NMR (D_2O)*
	C_{18}: 30.8
	C_{19}: 35.8
$H_{18,27}$:2.1;m;3H	C_{16}: 41.5
H_{19}: 2.5; t; 2H	C_{27}: 51.4 (t; $J_{C\text{-}P}$ = 121.5 Hz)
H_{16}: 3.1; s; 3H	C_9 : 57.5
H_{17}: 4.3; m; 1H	C_{17}: 58.2
H_9: 4.8; s; 2H	C_{13}: 131.6
H_{12}: 6.7; d; 2H	C_6 : 150.8
H_{13}: 7.7; d; 2H	C_7 : 151.9
H_7: 8.6; s; 1H	C_{11}: 154.4
	C_4 : 156.0
	C_2 : 164.6
^{31}P-NMR (D_2O)	C_{8a}: 165.4
	C_{15}: 172.1
P: 13.6; s	C_{20}: 176.9
	C_{21}: 181.7

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