JOM 23027

# Synthetic pathways for selective introduction of a dicobalt hexacarbonyl cluster into a polyfunctional molecule: methotrexate

M. Salmain and G. Jaouen

*Ecole Nationale Supérieure de Chimie de Paris – URA CNRS 403 – 11, rue Pierre et Marie Curie, 75231 Paris Cedex 05 (France)*

(Received March 25, 1992)

**Abstract**

For the development and applications of organometallic moieties as potential tracers for biological assays, several methods are explored for the labelling of a polyfunctional and nearly insoluble biomolecule with a dicobalt hexacarbonyl marker. This can be done successfully using an organometallic-like Bolton–Hunter reagent. The metal-carbonyl isomeric adducts are obtained in the expected proportions and are characterized by high performance liquid chromatography, IR spectroscopy and fast atom bombardment/mass spectroscopy.

## 1. Introduction

The last few years have seen the development of an interrelationship between organometallic chemistry and biochemistry. This area of research has given birth to a new topic of science called organometallic biochemistry which has been reviewed recently by Ryabov [1]. When considering transition metal organocomplexes as probes, a large variety of detection techniques can currently be utilized. Atomic absorption spectroscopy [2], electrochemical techniques [3] and isotopic counting [4] when the metal is radioactive, are the most often cited. As part of our research work, we have shown that organometallic chemistry can provide unique and new information concerning hormone/receptor interactions in the case of oestrogens [5]. Some applications for using organometallic markers as sensitive and specific biological probes in clinical chemistry have also been proposed [6]. In particular, we have suggested a new type of immunoassay called carbonylmetalloimmunoassay (CMIA) which associates metallocarbonyl tracers and Fourier transform IR (FT-IR) spectroscopy. This method has been successfully applied to drug [7] and cortisol [8] assays.

The main advantages of the CMIA method are chemical and spectroscopic. As there is formation of a covalent bond between the molecule and the probe, the resulting tracer is stable and retains good affinity for the target protein (receptor or antibody). Moreover, non-specific binding (between the tracer and the other proteins) is low. Because metal–carbonyl entities are purely artificial, the label can be detected spectroscopically unambiguously. Moreover, we have recently shown that dicobalt hexacarbonyl compounds can be detected quantitatively by FT-IR spectroscopy with a high sensitivity [9].

However, the use of organometallic bioprobes is currently limited by the type of molecule to be assayed. Only biological compounds soluble in organic solvents can be labelled in this manner [10]. We show in this article for the first time that a hydrophilic compound, methotrexate, can be labelled by a metal–carbonyl moiety using a few synthetic steps.

Methotrexate **1** (MTX) is an antineoplastic drug prescribed to children suffering from acute leukemia [11] and to adults for other cancerous pathologies [12]. The monitoring of its plasma level is crucial to avoid toxic side-effects associated with high doses [13]. We thought it interesting to show that a CMIA assay could be applied here, knowing that the concentrations to be measured (1 mg $l^{-1}$) are in the range of the sensitivity of this method. In this article we present a strategy for introduction of a dicobalt hexacarbonyl cluster into the molecule of methotrexate using an *N*-succinimidyl ester.

Correspondence to: Professor G. Jaouen.

0022-328X/93/$6.00 © 1993 – Elsevier Sequoia S.A. All rights reserved

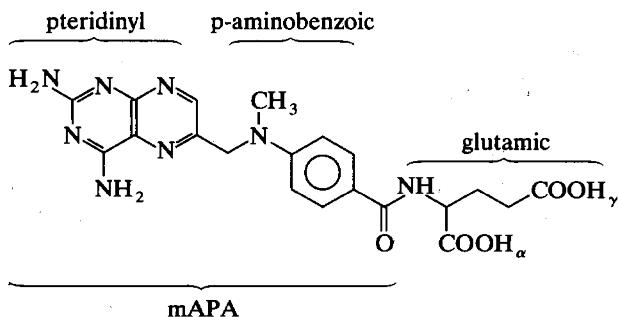
## 2. Results and discussion

The molecule of MTX (Scheme 1) can be divided into three parts: a pteridinyl part, a *p*-methylaminobenzoic part and a glutamic part. The first two parts are together designated by the symbol mAPA. The $^1$H nuclear magnetic resonance (NMR) spectrum (250 MHz) of MTX in dimethylsulphoxide (DMSO)-$d_6$ is reported in Table 1 and is consistent with the literature [14].

MTX is insoluble in common organic solvents (except DMSO) and slightly soluble in dilute acid and aqueous alkali media. p$K_a$ values for the ionizable functions are 5.32 and 0.5 for the amino groups [15] and 3.36 and 4.7 respectively for the $\alpha$ and $\gamma$ carboxylic functions [16].

The strategy of coupling of an organometallic marker to MTX must consider the mode of preparation of antibodies directed against this drug, the chemically modifiable functions, and the solubility. The main difficulty comes from the almost insoluble and polyfunctional character of MTX.

Anti-MTX antibodies are generally raised in animals after immunization with an antigen (or immunogen) resulting from the conjugation of the drug to a carrier protein from the two carboxylic functions of the glutamic part [17,18]. On the other hand, the relative insolubility of MTX restricts the number of solvents useful for the complexation of [$Co_2(CO)_8$]. Therefore, we planned to transform the carboxylic functions into organometallic amides by a peptidic type synthesis. The simplest way (Scheme 2) where [$Co_2(CO)_6$(propargylamine)] is conjugated to MTX, failed whatever the coupling reagent used. We suspect that the amine group is rendered less nucleophilic by complexation and therefore less reactive. On the other hand, the attachment of propargylamine to the $\alpha$ and $\gamma$ carboxylic groups of MTX in the presence of a carbodiimide was successful. Three adducts resulting from the coupling in $\alpha$, $\gamma$ and both $\alpha$ and $\gamma$ positions were expected. The mixture was analysed by reverse-phase (RP) high performance liquid chromatography (HPLC) which showed four peaks, at 4.25, 9.35, 10.15 and 14.05 min, the first being readily assigned to unreacted MTX. The four components (MTX, **2α**, **2γ** and **2αγ**) were separated by preparative HPLC and the isomers **2α** and **2γ** were assigned from the $^1$H NMR chemical shifts compared with MTX (Table 2). The first isomer eluted shows different chemical shift values for H 22, H 23 and H 24. The second isomer eluted shows a slightly different chemical shift for H 23 but not for H 22. From these observations, we conclude that the first is the $\gamma$ isomer and the second the $\alpha$.




Scheme 1. MTX **1**.

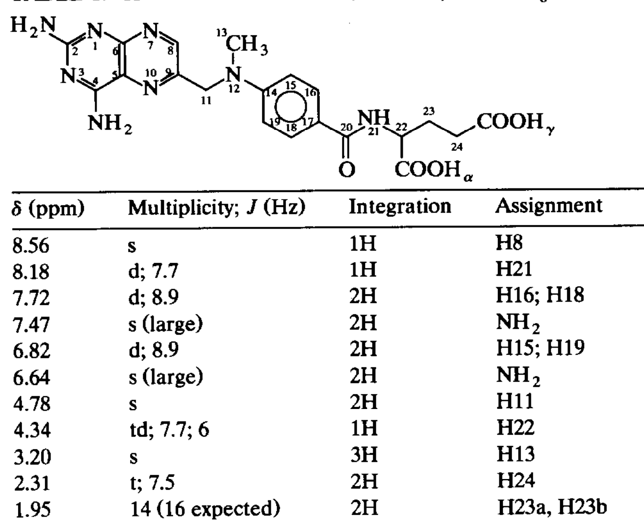
Two complexation methods using unusual solvents have been described in the literature [19,20]. The complexation of a mixture of **2α** and **2γ** by [$Co_2(CO)_8$] was tried using the method described by Le Borgne and Beaucourt for the synthesis of $Co_2(CO)_6$-labelled peptides using a methanol/acetic acid mixture as the solvent, at low temperature to minimize the metal–carbonyl decomposition. Instead of the expected adducts, we obtained a very unstable complexation product which lacked the pteridinyl and aromatic groups, indicating a probable hydrolysis of the glutamic moiety during the reaction.

Since $Co_2(CO)_6$ cannot be introduced directly into MTX, we tried introducing it indirectly, complexed to an alkyne group. In a recent publication, we described the preparation of a Bolton–Hunter-like reagent bearing a metallo–carbonyl group and an example of its condensation with a functionalized derivative of carbamazepine in an organic medium [21]. This reaction takes advantage of the selectivity of *N*-succinimidyl esters for amino groups, leading to the formation of a peptide bond.

This strategy, B, involves introduction of a primary amino group into MTX by condensation with ethylenediamine. Method B1, introduced by Williams [22] and already used for the synthesis of 2, involves adding to the mixture of acid and amine, a water-soluble carbodiimide, 1-ethyl 3-[3-(dimethylamino)propyl]-carbodiimide, HCl, or EDAC. Method B2, also called "mixed anhydride", was introduced by Erlanger *et al.* and is carried out by adding isobutylchloroformate (IBCF) in the presence of triethylamine (TEA) [23]. Methods B1 and B2 gave three condensation adducts, **3α**, **3γ** and **3αγ**. The analysis of the proportions of each compound was performed by RP HPLC. Percentages of each product were calculated from peak areas (detection wavelength 304 nm), and **3α** and **3γ** isomers assigned by comparison of the elution order with that of **2α** and **2γ** isomers (Table 3).

Methods B1 and B2 led to yields of 67% and 89% respectively. The latter is higher probably because of the excess of reagents. Method B1 gave preferentially **3γ** (as for the condensation of propargylamine; experiment 1), but with a longer reaction time, proportions of

TABLE 1. $^1H$ NMR data for MTX (250 MHz, DMSO-$d_6$)

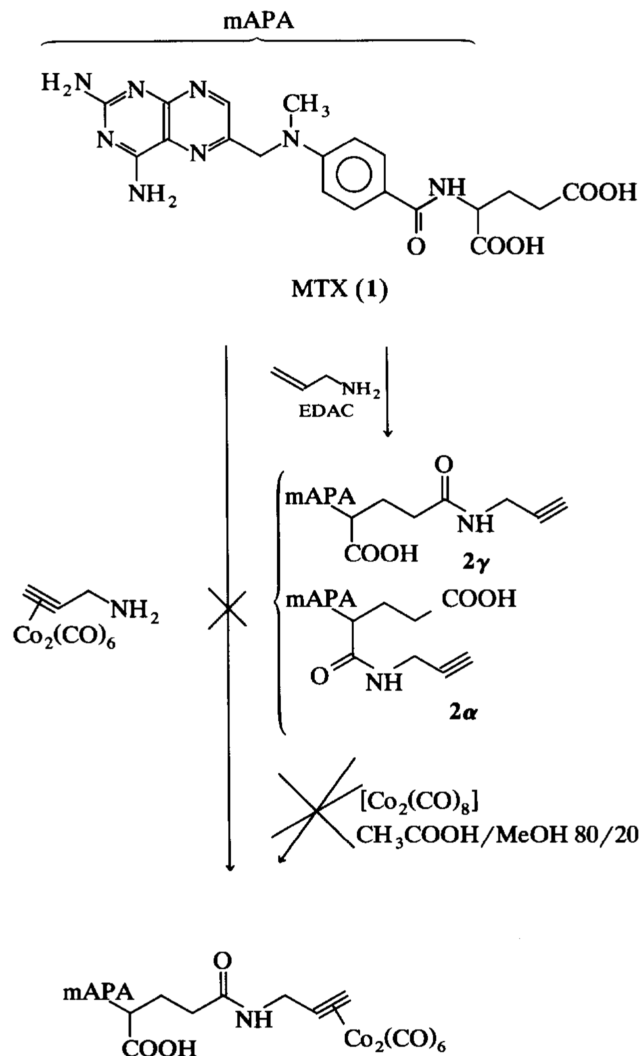


| δ (ppm) | Multiplicity; $J$ (Hz) | Integration | Assignment |
|---|---|---|---|
| 8.56 | s | 1H | H8 |
| 8.18 | d; 7.7 | 1H | H21 |
| 7.72 | d; 8.9 | 2H | H16; H18 |
| 7.47 | s (large) | 2H | $NH_2$ |
| 6.82 | d; 8.9 | 2H | H15; H19 |
| 6.64 | s (large) | 2H | $NH_2$ |
| 4.78 | s | 2H | H11 |
| 4.34 | td; 7.7; 6 | 1H | H22 |
| 3.20 | s | 3H | H13 |
| 2.31 | t; 7.5 | 2H | H24 |
| 1.95 | 14 (16 expected) | 2H | H23a, H23b |

**3α** and **3γ** are more equal (experiment 2). Method B2, after equilibration, gave **3α** in an excess (experiment 4), although **3γ** seems to be formed faster in the medium (experiment 3). Thus, either isomer **3α** or **3γ** was obtained in excess. The difference in acidities for α and γ carboxylic functions may explain this selectivity. Rosowsky and coworkers [24,25] underlined the relative instability of the α-monomethyl ester of MTX which rapidly transforms into the γ ester of MTX, probably because the α COOH function is more acidic than the γ. Under these conditions (slightly acidic medium), yields of the γ monoesters were three to five times higher than of the α isomers [24,25]. Under our conditions, an acidic medium (method B1) favours the formation of **3γ** and an alkaline medium favours the α isomer.

The mixture of **3α** and **3γ** isomers from experiment 2 was allowed to react with hexacarbonyl ($N$-succinimidyl-4-pentynoate) dicobalt **4** in aqueous/organic medium and the kinetics were followed by analytical RP-HLPC. The formation of one of the expected products was shown by the appearance of a peak at $t_R = 6.6$ min. At the same time, the 4.3 min (**3γ**) peak gradually disappeared. The ratio of areas of these two peaks as a function of reaction time (Fig. 1) shows that the reaction is complete in about 5 h. After extraction of the excess of **4** using chloroform, the aqueous phase was chromatographed on a silica gel column and the product obtained was characterized by IR spectroscopy and fast atom bombardment/mass spectroscopy (FAB/MS.)

An HPLC analysis of an acidic solution of the mixture of **5** isomers was then performed (Fig. 2). Three fractions corresponding to the three major peaks were recovered and analysed by IR spectroscopy in the ν(CO) region. Only fractions 1 ($t_R = 7.50$ min) and 2 ($t_R = 8.86$ min) showed bands corresponding to an ($[RC{\equiv}CR')Co_2(CO)_6]$ adduct. By analogy with the elution order of **2α** and **2γ**, fractions 1 and 2 were assigned to **5γ** and **5α**. In Table 4, we report absorption data together with the concentration of the fractions calculated from the standard curve Fig. 3 [26]. The percentages of **5α** and **5γ** calculated this way are identical to those of **3α** and **3γ** from which **5** was synthesized (see Table 3, experiment 2). The separation of larger quantities of **5α** and **5γ** isomers was performed on a silica gel thin-layer chromatography (TLC) plate from an acidic solution of the mixture **5**, using 0.1% of trifluoroacetic acid (TFA) in the eluent to improve the separation. A FAB/MS analysis of the major isomer gave the expected molecular mass. No




Scheme 2. Strategy A.

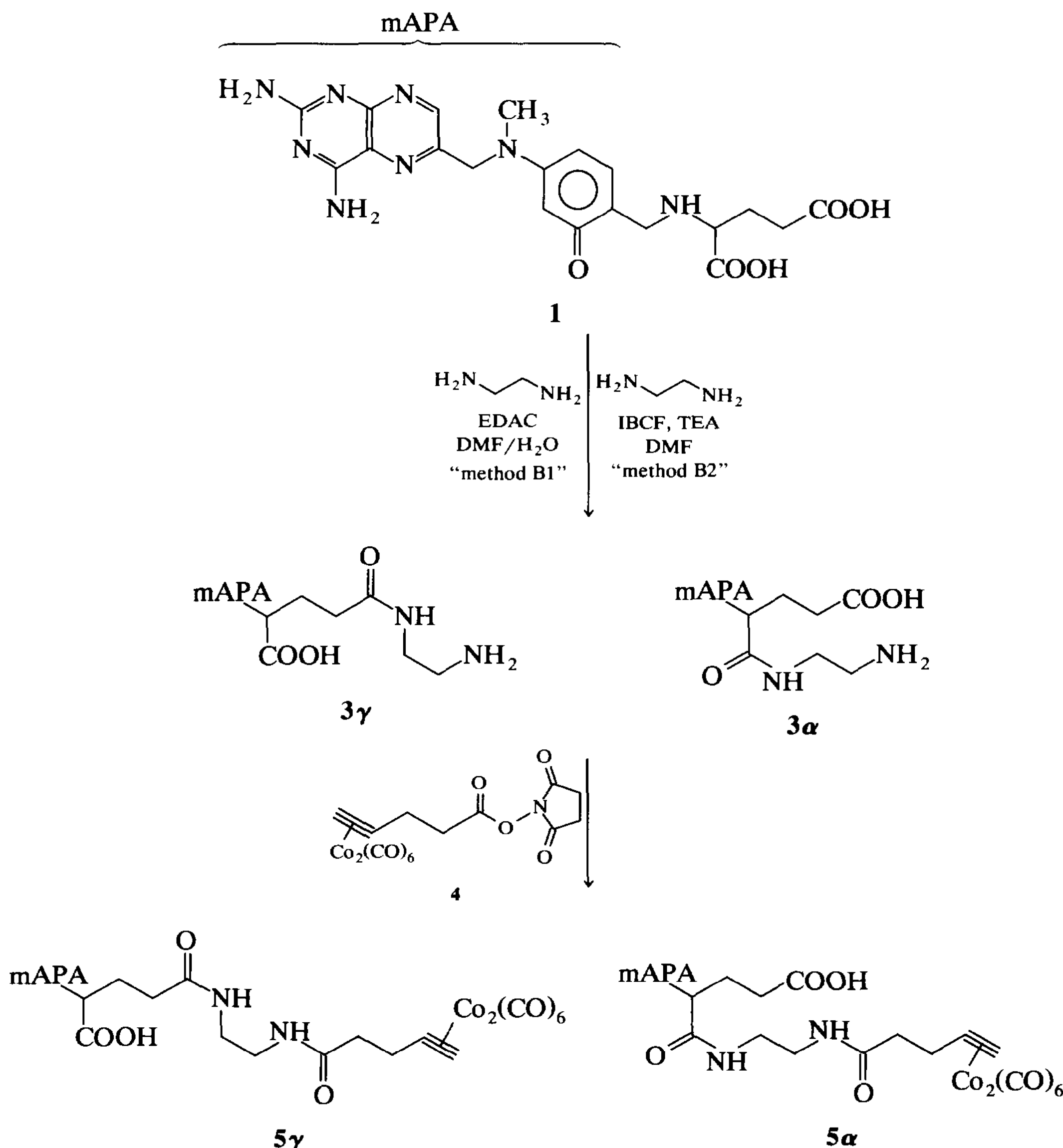
clear difference in the MS spectra of the $\alpha$ and $\gamma$ isomers was observed.

## 3. Conclusion

The labelling of a polyfunctional, nearly insoluble molecule by a metal–carbonyl cluster was achieved in two steps, in a partially aqueous medium and without decomplexation, with a Bolton–Hunter-like reagent. A primary amino group was introduced during the first step using two different methods. Two monoamino isomers were obtained but in different proportions depending on the method. In each case, a different isomer was obtained in excess, indicating good overall selectivity. This mixture reacted with the metallocarbonyl Bolton–Hunter reagent which led to the two isomeric organometallic adducts expected, in the same proportions. An infrared analysis of the two isomeric adducts in the $\nu$(CO) region shows the expected characteristic bands.

## 4. Experimental details

*d,l*-Methotrexate was obtained from Rhône-Poulenc Specia, other chemicals were purchased from Aldrich (Strasbourg, France). Solvents were purchased from Prolabo (Paris, France). NMR spectra were recorded at 250 MHz with a Bruker AM 250 deuterium lock. IR spectra were recorded on a Bomem Michelson 100, 4 $cm^{-1}$ resolution with a DTGS detector (full range) or




Scheme 3. Strategy B.

TABLE 2. $^{1}H$ NMR data for **2α** and **2γ** (DMSO-$d_6$). H corresponding to the propargylamide are bold. Shifts in italics different from corresponding resonances of MTX

| δ (ppm) | Multiplicity; J (Hz) | Number of H | Assignment |
|---|---|---|---|
| *α isomer* | | | |
| 8.56 | s | 1H | H8 |
| **8.30** | **t; 4.75** | **1H** | **H25** |
| 8.23 | d; 7.57 | 1H | H21 |
| 7.72 | d; 8.0 | 2H | H16, H18 |
| 7.48 | s (large) | 2H | NH2 |
| 6.82 | d; 8.0 | 2H | H15, H19 |
| 6.63 | s (large) | 2H | NH2 |
| 4.78 | s | 1H | H11 |
| *4.27* | *m* | *1H* | *H22* |
| **3.82** | **m** | **2H** | **H26** |
| 3.21 | s | 3H | H13 |
| **3.07** | **t; 1.5** | **1H** | **H27** |
| *2.20* | *t; 7.1* | *2H* | *H24* |
| *2.15* | *m* | *2H* | *H23* |
| *γ isomer (selected values)* | | | |
| **8.34** | **t; 4** | **1H** | **H25** |
| *8.10* | *d; 8.47* | *1H* | *H21* |
| 4.37 | m | 1H | H22 |
| **3.83** | **m** | **2H** | **H26** |
| **3.07** | **t; 2.6** | **1H** | **H27** |
| *2.23* | *m* | *2H* | *H24* |
| 1.93 | m | 2H | H23 |

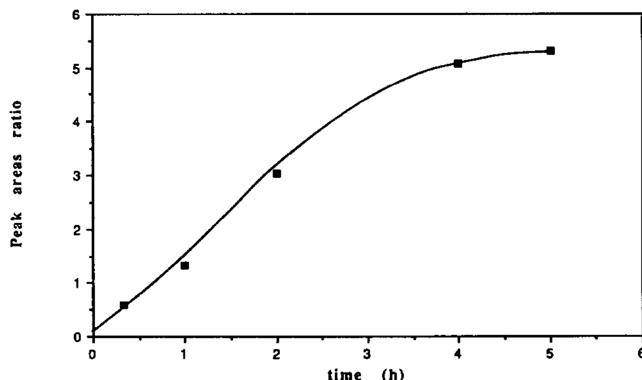



Fig. 1. Kinetics of reaction of **3** with **4** followed by HPLC. 20 μl of the mixture was periodically injected. Elution conditions: RP C8 column 150×4.6 mm², 5 μm. Eluant A, water; eluant B, methanol. Linear gradient from 30% of B to 100% of B over 10 min. Flow rate: 1 ml min⁻¹; UV 304 nm; area of 6.6 min peak was ratioed to area of 4.3 min peak.

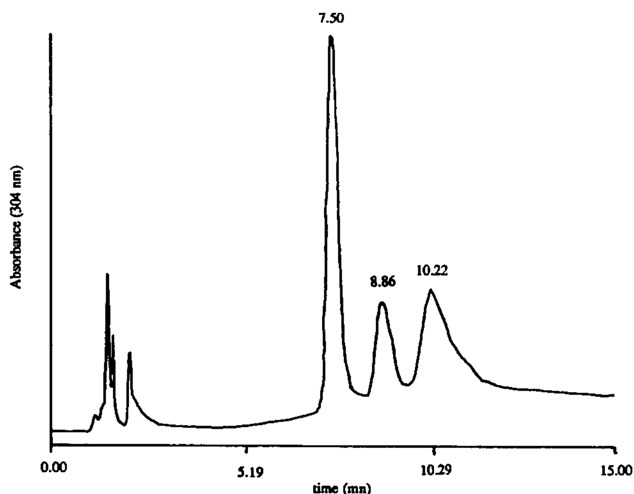



Fig. 2. Chromatogram of the mixture of **5** isomers. RP C8 150×4.6 mm, 5 μm. Eluant A, water; eluant B, methanol. Linear gradient from 30% of B to 100% of B over 10 min then 100% of B. Flow rate: 1 ml min⁻¹; UV 304 nm. Retention times: **5α** = 8.86 min; **5γ** = 7.50 min.

TABLE 3. Percentages of **3α**, **3γ** and **3αγ** obtained with methods B1 and B2. Results calculated from areas of chromatographic peaks. For elution conditions, see experimental details

| Compounds | MTX | 3γ | 3α | 3αγ |
|---|---|---|---|---|
| Retention time (min) | 2.3 | 3.7 | 5.5 | 8.3 |
| Experiment No. | 1 | 2 | 3 | 4 |
| Method | B1 | B1 | B2 | B2 |
| Reagents quantity | 1.1 eq. | 1.6 eq. | 2 eq. | 2 eq. |
| Reaction time | 3 h | 6 h | 0.5 h | 4 h |
| % MTX | 33 | 33 | 11 | 11 |
| % **3γ** | 56 | 43 | 50 | 12 |
| % **3α** | 7 | 23 | 32 | 73 |
| % **3αγ** | 4 | 0.5 | 5 | 4 |
| Ratio γ/α(%) | 89/11 | 65/35 | 61/39 | 14/86 |

TABLE 4. IR absorption of **5α** and **5γ** in the ν CO region of the spectrum (3 mm diameter KBr pellet from 20 μl of each fraction deposited on 15 mg of KBr powder and evaporated under vacuum, spectra recorded with an InSb detector)

| | ν (CO) (cm⁻¹) | Absorbance | Concentration | % isomer |
|---|---|---|---|---|
| Fraction 1 (**5γ**) | 2091 | – | | |
| | 2051 | 0.010 | 2×10⁻⁵ M | 63 |
| | 2022 | – | | |
| Fraction 2 (**5α**) | 2090 | 0.00126 | | |
| | 2051 | 0.00583 | 1.3×10⁻⁵ M | 37 |
| | 2021 | 0.00446 | | |

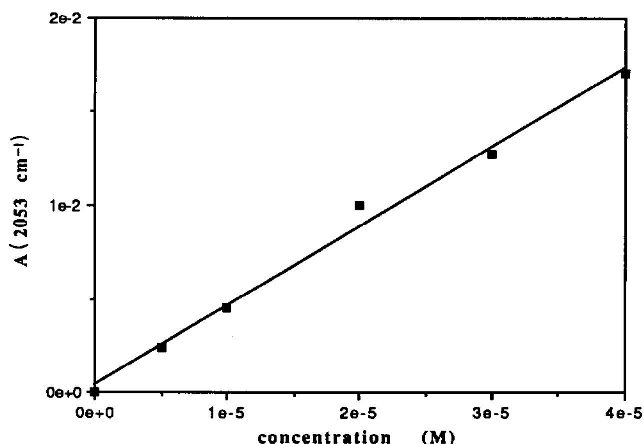



Fig. 3. Standard plot of absorbance *vs.* concentration for a complex $[Co_2(CO)_6(RC{\equiv}CR^1)]$ (3 mm KBr pellet, $\nu = 2053$ $cm^{-1}$, see ref. 26 for experimental details.

an InSb detector (1800–4000 $cm^{-1}$). Melting points were determined on a Köpfler stage. Preparative chromatography was performed with Merck Kiesel gel 60 contained in a glass column (5 × 1.7 cm), or else on Kieselgel 60 F254 TLC plates (0.2 mm thick). Analytical (150 × 4.6 mm columns) and preparative (250 × 10 mm column) RP-HPLC were performed with a Beckman System GOLD equipped with a 126 pump and a 166 UV detector coupled with an automatic fractions collector (Eurosas, France). FAB/MB spectra were recorded in a thioglycerol + HCl matrix at ICSN (Gif/Yvette, France).

### 4.1. *N-[4-{[(2,4-diamino-6-pteridinyl)-methyl]methylamine}benzoyl]-(1-propargylamide) glutamic acid 2*

To 226 mg (0.5 mmol) of $MTX.H_2O$ in 30 ml of deionized water, were added successively 95 mg of EDAC (0.5 mmol) and 0.035 ml (0.05 mmol) of propargylamine. The pH was adjusted to 5.5 and the mixture was incubated for 4 h at 4°C. The solution was then neutralized and extracted with diethyl ether. A mixture of **1** and **2** was precipitated by addition of methanol to the aqueous phase and the resulting yellow powder (280 mg) was dried under vacuum. It was further analysed by HPLC with a RP C18 column, 5 μm. Mobile phase: A, phosphate buffer; B, methanol. Linear gradient from 30% of B to 50% of B over 10 min then 50% of B. Flow rate: 1 ml $min^{-1}$, UV 304 nm. Retention times: MTX = 4.25 min; **2γ** = 9.35 min; **2α** = 10.15 min; **2αγ** = 14.05 min. **2α** m.p. 197 °C dec. **2γ** m.p. 155 °C dec.

IR (KBr, $cm^{-1}$) 1648 (amide I) 1603 1362 1305.

UV–VIS (NaOH 0.01 M, λ in nm, ε in l $mol^{-1}$ $cm^{-1}$), 371, 6 920; 304, 21 400; 256, 20 900; 224, 17600.

### 4.2. *N-[4-{[(2,4-diamino-6-pteridinyl)-methyl]methylamine}benzoyl]-(2-aminoethylamide) glutamic acid 3*

#### 4.2.1. *Method B1 (experiment 2)*

To 100 mg (0.22 mmol) of MTX in 5 ml of DMF was added 64 mg (0.34 mmol; 1.6 eq.) of EDAC in 1 ml of phosphate buffer (pH = 7.4; 0.1 M). After 0.5 h, 0.025 ml (0.38 mmol) of ethylene diamine in 2 ml of phosphate buffer was added. The mixture was incubated for 6 h at 4 °C. The precipitate was centrifuged, washed three times with methanol and was allowed to dry in air. The yellow powder was analysed by HPLC on a RP C8 column 150 mm × 4.6 mm, 5 μm. Mobile phase: A, phosphate buffer; B, methanol. Linear gradient from 30% of B to 50% of B over 10 min. Flow rate: 1 ml $min^{-1}$; UV 304 nm. Retention times (min): MTX = 2.31; **3γ** = 3.95; **3α** = 5.57; **3αγ** = 8.38.

$^1H$ NMR (DMSO-$d_6$, δ in ppm): **3α** isomer: 8.546 (s, H8) 8.23 (d, $J = 8.2$ Hz, H21) 7.75 (d, $J = 9.5$ Hz, H6 and H18) 7.46 (s, $NH_2$) 6.82 (d, $J = 8.9$ Hz, H15 and H19) 6.63 (s, $NH_2$) 4.78 (s, H11) 4.20 (m, H22) 3.21 (s, H13) 2.84 (m, $CH_2$–$NH_2$) 2.30 (m, H24) 2.13 (m, H23) 1.23 (s, $CH_2$–$NH_2$). **3γ** isomer: 8.56 (s, H8) 8.10 (d, $J = 8.2$ Hz, H21) 7.69 (d, $J = 8.8$ Hz, H16 and H18) 7.46 (s, $NH_2$) 6.82 (d, $J = 8.9$ Hz, H15 and H19) 6.63 (s, $NH_2$) 4.78 (s, H11) 4.34 (m, H22) 3.20 (s, H13) 2.84 (m, $CH_2$–$NH_2$) 2.30 (m, H24) 1.95 (m, H23) 1.14 (s, $CH_2$–$NH_2$).

#### 4.2.2. *Method B2 (experiment 3)*

MTX (200 mg (0.44 mmol)), previously dried in a desiccator containing $P_2O_5$, was suspended in 5 ml of dry dimethyl formamide (DMF) and 0.07 ml (1 mmol, 2 eq.) of TEA and 0.065 ml of isobutylchloroformate (IBCF) (1 mmol) were added. The solution became clear after 5 min. After another 20 min, 0.05 ml (0.75 mmol) of ethylenediamine was added. The formation of a yellow precipitate occurred immediately. The mixture was stirred for 0.25 h and the precipitate was recovered after centrifugation and several washes with chloroform.

### 4.3. *Hexacarbonyl (N-succinimidyl-4-pentynoate) dicobalt 4*

**4** was synthesized according to the literature procedure [21].

### 4.4. *N-[4-{[(2,4-diamino-6-pteridinyl)-methyl]methylamine}benzoyl](2-[4-pentyn{dicobalthexacarbonyl}amido]ethylamide) glutamic acid 5*

16 mg of **3** was dissolved in 2 ml of borate buffer (pH 9.6; 0.1 M) and cooled in an ice bath. **4** dissolved in 2 ml of methanol was added slowly and the mixture was stirred for 5 h at 4°C. Unreacted **4** was extracted

by chloroform and the aqueous phase was evaporated under vacuum. The residue was dissolved in methanol and chromatographed on a silica gel column with chloroform/methanol 1/1 as the eluant. After 20 min, an orange fraction was collected and the solvent evaporated under vacuum. The residue was dissolved in methanol and a red powder was obtained by addition of diethyl ether. HPLC analysis, see Fig. 3.

IR (KBr, $cm^{-1}$, Fig. 2) 2094, 2051, 2020 ($\nu$ (MCO)), 1635 (amide I), 1607 (aromatic C–C), 1558 (amide II), 1508, 1448 (aromatic C–C), 1208 ($\nu_a$ (C–O–C), 1103 ($\nu_s$ (C–O–C)), 827 ($\delta$(C–H aromatic)).

FAB/MS: $MH^+$, 863; MNa, 885; $MH^+$–6 CO, 695; MNa–6 CO, 717; M–$Co(CO)_6$, 635.

The mixture of **5α** and **5γ** was dissolved in MeOH containing 0.1% of TFA and chromatographed on a silica gel plate with $CH_2Cl_2$/MeOH 1/1 containing 0.1% of TFA. **5γ**: $R_f = 0.8$; **5α**: $R_f = 0.94$.

IR (KBr, $cm^{-1}$) **5γ**: 2091, 2051, 2022; **5α**: 2090, 2051, 2021 (all $\nu$(CO)).

FAB/MS. **5γ**: $MH^+$, 863; MNa, 885; $MH^+$–4 CO, 751; $MH^+$–6 CO, 695; MNa–6 CO, 717; M–$Co(CO)_6$, 635.

## Acknowledgments

We thank CNRS and région Bourgogne for financial support, and Specia Rhône-Poulenc for methotrexate. We also thank Professor James Howell for his efficient correction of the manuscript and P. Brossier for helpful discussion. We finally wish to thank B. Das from ICSN Gif/Yvette, France for the FAB/MS analysis.

## References


1 A. D. Ryabov, *Angew. Chem., Int. Ed. Engl., 30* (1991) 931.

2 (a) M. Cais, E. Slovin and L. Snarsky, *J. Organomet. Chem., 160* (1978) 223; (b) M. Cais, *Methods Enzymol., 70* (1980) 336; (c) P. Chéret and P. Brossier, *Res. Comm. Chem. Pathol. Pharmacol., 54* (1986) 237; (d) F. Mariet and P. Brossier, *Res. Comm. Chem. Pathol. Pharmacol., 68* (1990) 251.

3 (a) S. G. Weber and W. C. Purdy, *Anal. Lett.*, 12 (1979) 1; (b) K. Di Gleria, H. Allen, O. Hill, J. McNeil and M. J. Green, *Anal. Chem., 58* (1986) 1203.

4 (a) B. Risselmann, M. Wenzel and S. Hoppe-Seyler, *Z. Physiol. Chem.*, (1977) 1353; (b) K. E. Linder, M. D. Wen, D. P. Nowotnik, M. F. Malley, J. Z. Gougoutas, A. D. Nunn and W. C. Eckelman, *Bioconjugate Chem., 2* (1991) 160.

5 (a) A. Vessières, G. Jaouen, M. Gruselle, J. L. Rossignol, M. Savignac, S. Top and S. Greenfield, *J. Steroid Biochem., 30* (1988) 301; (b) A. Vessières, S. Top, A. A. Ismail, I. S. Butler, M. Loüer and G. Jaouen, *Biochemistry, 27* (1988) 6659.

6 (a) M. Cais, S. Dani, Y. Eden, O. Gandolfi, M. Horn, E. E. Isaacs, Y. J. Josephi, Y. Saar, E. Slovin and L. Snarsky, *Nature, 270* (1977) 534; (b) G. Jaouen, A. Vessières, S. Top, A. A. Ismail and I. S. Butler, *J. Am. Chem. Soc., 107* (1985) 4778; (c) M. Gruselle, V. Philomin, F. Chaminant and K. M. Nicholas, *J. Organomet. Chem., 399* (1990) 317.

7 M. Salmain, A. Vessières, P. Brossier, I. S. Butler and G. Jaouen, *J. Immunol. Methods, 148* (1992) 65.

8 A. Vessières, M. Salmain, V. Philomin and G. Jaouen, *Immunoanal. Biol. Spéc., 31* (1991) 9.

9 M. Salmain, A. Vessières, G. Jaouen and I. S. Butler, *Anal. Chem., 63* (1991) 2323.

10 I. Lavastre, J. Besançon, P. Brossier and C. Moise, *App. Organomet. Chem., 5* (1991) 143.

11 (a) S. Farben, L. K. Diamond and R. D. Mercer, *N. Engl. J. Med., 238* (1948) 787. (b) I. Djerassi, *Cancer Res., 27* (1967) 2562.

12 K. Kimura and Y. M. Wong, *Methotrexate in Cancer Therapy*, Raven Press, New York, 1986.

13 B. A. Charbner, N. Clendeninn, G. A. Curt and J. Jolivet, in K. Kimura and Y. M. Wong (eds.), *Methotrexate in Cancer Therapy*, Raven Press, New York, 1986, p. 1.

14 A. R. Chamberlain, in K. Florey (ed.), *Analytical Profiles of Drug Substances*, Vol. 5, Academic Press, New York, 1976, p. 286.

15 A. Albert, D. J. Brown and G. Chessman, *J. Chem. Soc.*, (1952) 4219.

16 M. Poe, *J. Biol. Chem., 252* (1977) 3724.

17 R. S. Kamel and J. Landon, *Methods Enzymol., 84* (1982) 422.

18 M. C. Cot, *Ph. D. thesis*, Montpellier, 1986.

19 C. Sergheraert, J. C. Brunet and A. Tartar, *J. Chem. Soc., Chem. Commun.* (1982) 1417.

20 F. Le Borgne and J. P. Beaucourt, *Tetrahedron Lett., 29* (1988) 5644.

21 M. Salmain, A. Vessières, G. Jaouen and I. S. Butler, *Bioconjugate Chem., 2* (1991) 13.

22 D. G. Williams, in S. D. Pal (ed.), *Enzyme Labelled Immunoassay of Hormones and Drugs*, Walter de Gruyter, New York, 1970, p. 129.

23 B. F. Erlanger, F. Borek and S. M. Beiser, *J. Biol. Chem., 228* (1957) 713.

24 A. Rosowsky, G. P. Beardsley, W. D. Ensminger, H. Lazarus and C.-S. Yu, *J. Med. Chem., 21* (1978) 380.

25 A. Rosowsky and C.-S. Yu, *J. Med. Chem., 21* (1978) 170.

26 I. S. Butler, A. Vessières, M. Salmain, P. Brossier and G. Jaouen, *Appl. Spectrosc., 43* (1989) 1497.