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Preliminary communication

Synthesis and biological evaluation of Fotemustine analogues on human melanoma cell lines

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Abstract

Two new analogues of Fotemustine have been synthesized and tested on two melanoma cell lines. Compounds **4** and **8** proved to be more potent than the reference compound on A375 cell line which express the MGMT enzyme involved in the chemoresistance of tumoral cells.

Keywords: Fotemustine; Nitrososulfamide; Melanoma; chemoresistance

1. Introduction

Among antitumor agents, nitrosoureas [1] are an extremely active class of alkylating compounds that have widespread clinical application in the treatment of brain tumours, melanomas and various leukemias [2]. The clinical application of these anticancer agents, which exhibit their cytotoxicity due to their alkylating properties, is, however, limited by their toxic side effects such as leucopenia and above all, thrombocytopenia [3]. The therapeutic efficacies of nitrosoureas are known to be related to their spontaneous decompositions to generate both electrophilic species which alkylate DNA and isocyanates which carbamoylate proteins especially DNA repair proteins, contributing substantially to toxic side effects [4].

On the basis of these observations, we previously reported a new family of alkylating agents structurally related to 2-chloroethylnitrosoureas (CENU) but devoid of any carbamoylating activity: 2-chloroethylnitrososulfamides (CENS) [5].

A certain number of these derivatives exhibited interesting cytotoxic activity and among them, some proved to be considerably more potent than the parent nitrosourea [5].

Fotemustine [6] (Muphoran®) is a third generation nitrosourea which is used clinically against disseminated malignant melanoma [7]. However its clinical application is somewhat limited by its toxicity [8] and also by acquired resistance of melanoma cells to this antineoplastic agent [9].

In the present paper we report the synthesis of sulfamides analogues of Fotemustine (Fig. 1) and their preliminary in vitro evaluation on two human melanoma cell lines.

2. Chemistry

The synthetic pathway used for the preparation of the different analogues is outlined in Fig. 2. The starting material diethyl aminomethylphosphonate **1** was readily available on reduction of the corresponding 1-hydro-

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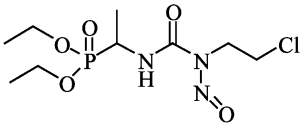
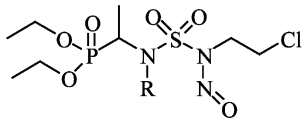


Fotemustine



R= -CH_3, -$(CH_2)_2Cl$

Sulfamides analogues

Fig. 1. Fotemustine and its synthesized analogues.

xyiminophosphonate according to a method reported in literature [10].

Sulfamoylation of **1** was carried out using *tert*-butoxysulfamoyl chloride as reagent (prepared ab initio by reacting chlorosulfonylisocyanate (CSI) on *tert*-butanol), leading to **2** in 52% yield. This precursor was reacted with 2-chloroethanol under Mitsunobu conditions to afford products **3** and **5** as mixture in a 1/3–2/3 ratio, respectively. Removal of the *tert*-butoxycarbonyl group from **5** under acidic conditions produced the intermediate sulfamide in 100% yield, which was then nitrosated with sodium nitrite in formic acid. Compound **7** was produced in quantitative yield (TLC) but was not enough stable to be isolated in pure form. (The loss of nitroso group with recovery of the starting material was observed on TLC and by ^{31}P-NMR.) So in view of a better stability, an N-methylation of **5** was performed to furnish **6** in 97% yield. It is worth to point out that methylation of the nitrogen atom does not affect the activity of the final CENS compared to the non methylated product [5]. Cleavage of Boc protection and nitrosation provided **8** as yellow oil. In the same way, target compound **4** was produced as a single product from **3**. All samples were characterized by electrospray mass spectrometry, ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopies, TLC and CHN analysis prior to evaluation.

3. Biological assays

The new agents **4** and **8** have been evaluated in vitro against two human melanoma cell lines (A375 and CAL77) in comparison with Fotemustine.

3.1. Cell lines

Human melanoma cell lines A375 and CAL77 were cultured at 37 °C in a fully humidified 5% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g l^{-1} glucose,

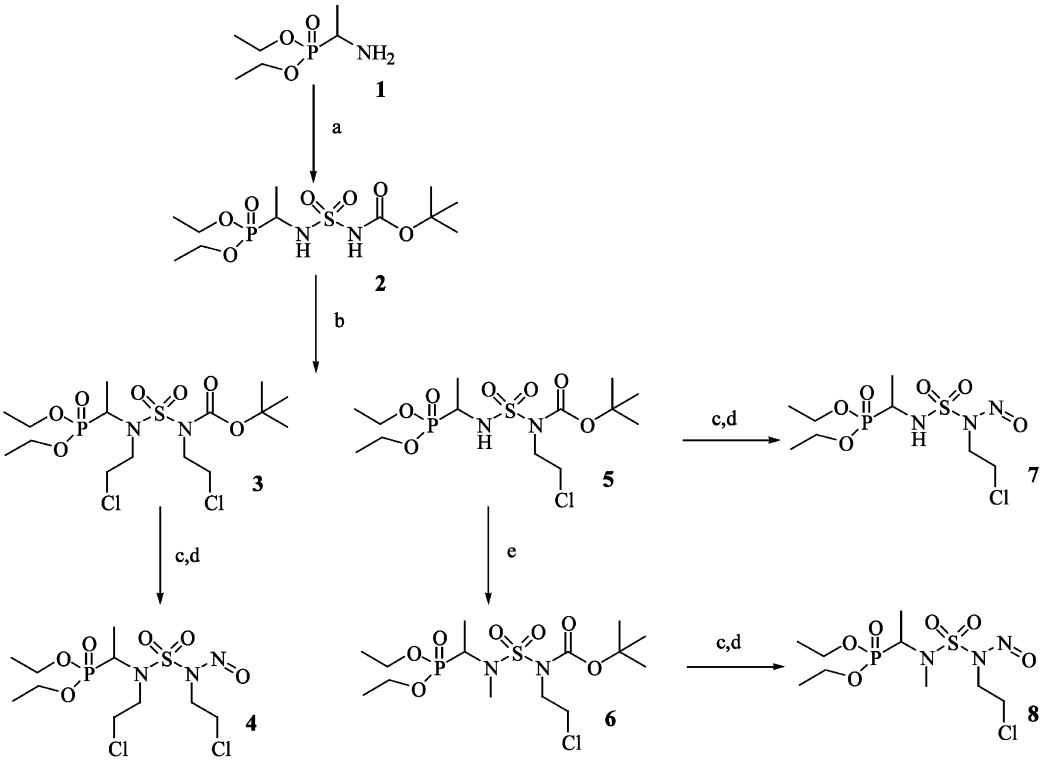


Fig. 2. Synthesis of Fotemustine analogues. Reagents and conditions: (a) *t*-BuOH, CSI, CH_2Cl_2, 0 °C; (b) PPh_3, DIAD, 2-chloroethanol, r.t.; (c) TFA 20% CH_2Cl_2, r.t.; (d) $NaNO_2$, HCOOH, 0 °C; (e) CH_3I, K_2CO_3, acetone, r.t.

10% fetal calf serum (FCS) and 2 mM glutamine. A375 cell line was from American Type Cell Culture Collection (ATCC, CRL-1619) and CAL77 cell line [11] was from Centre Antoine Lacassagne (Nice, France). Both cell lines were checked for expression of standard melanoma antigens (Tyrosinase, Melan-A and NA-17).

3.2. Cell sensitivity assays

3.2.1. Neutral red assay A

Cells were seeded in 96-well microtiter plates at 5×10^3 cells per well for A375 and 10×10^3 cells per well for CAL77. After 24 h incubation, cells were treated for 1h with increasing concentrations (0–1000 μM) of Fotemustine analogues (150 μl in fresh medium per well, six wells per concentration). Drug containing medium was then replaced by fresh medium and cells were allowed to grow for 72 h. Thereafter, cells were washed with PBS and 150 μl of a neutral red solution (40 μg ml^{-1}) was added. After 3 h at 37 °C, cells were washed with PBS and destained with 150 μl of glacial acetic acid (1%)–ethanol (50%) (v/v). Absorbances at 540 nm (A_{540}) were measured using a microplate reader (Labsystems Multiscan MS). The effect of drugs on cell survival was expressed as a percentage of cell viability relative to untreated cells and IC_{50} (concentrations leading to a growth inhibition of 50%) were obtained from cytotoxicity curves.

3.2.2. Clonogenic assay B

A375 and CAL77 were plated at a density of 1.4×10^6 cells in 75 cm^2 flasks. After 24 h incubation, cells were treated with various concentrations (0, 50, 100 and 200 μM) of Fotemustine analogues for 1 h. Drug containing medium was then replaced by fresh medium and cells were allowed to grow for 16 h. Cells were then harvested by trypsinization, counted end replated at 10×10^3 cells per 60 cm^2 Petri dishes. After a 10–12 days incubation period, cell colonies were stained with Giemsa and automatically counted using Bio1D® image analysis software (Vilber Lourmat, France). The loss of colony-forming ability were evaluated for each drug concentration and expressed as a percentage relative to untreated cells.

4. Results and discussion

Antiproliferative activity of Fotemustine analogues **4** and **8** on melanoma cells were assessed using both a standard neutral red uptake assay [12] and a clonogenic assay [13]. These assays have allowed us on one hand to determine the IC_{50} of each drug and on the other hand to evaluate the ability of cells to form colonies after drug treatment (Fig. 3).

Compounds **4** and **8** have shown an in vitro activity three fold higher ($IC_{50} = 75$ μM) than the reference compound Fotemustine ($IC_{50} = 220$ μM) on A375 cell line. Results obtained with Fotemustine are consistent with those previously reported in literature [12].

A375 is a melanoma cell line which express DNA repair enzyme MGMT[1] (Mer^+ phenotype). MGMT repairs alkylation on the O^6-guanine site and is responsible for protecting both tumor and normal cells from alkylating agents. So this enzyme is believed to be involved in the chemoresistance of tumoral cells against nitrosoureas, and in particular Fotemustine [9]. The results obtained on A375 suggest that MGMT expression does not modify the activity of compounds **4** and **8**.

In order to confirm these observations, we tested **4** and **8** on CAL 77, a cell line which does not express MGMT (Mer^- phenotype). In this case, **4** and **8** showed to be less potent than the reference compound.

Considering than approximately 75–80% of melanomas are Mer^+, the enhanced activity observed on A375 cell line with compounds **4** and **8** is promising.

With the aim to evaluate the influence of MGMT expression on the efficacy of **4** and **8**, we carried out cytoxicity assays on a CAL 77 cell line which was transfected with MGMT cDNA to overexpress this enzyme. Preliminary results have displayed that overexpression of MGMT lead to chemoresistance to Fotemustine whereas sensitivity to compounds **4** and **8** is retained. Complementary study is currently in progress and will be reported in due course.

5. Experimental

5.1. Chemistry

Melting points (not corrected) were measured on a Buchi 510. NMR spectra were recorded with Bruker AC 250, or AMX 400 instruments. Electrospray mass spectrometry (ESIMS) were recorded in the positive-ion mode with Water Micromass ZQ. Carlo Erba silica gel 60 (35–70 μm) was used for purification by column chromatography. Thin layer chromatography (TLC) was performed on aluminium plates coated with silica gel 60 F254 (Merck). Ninhydrin (5% EtOH solution) or molybden blue solution were used to develop the plates. Dry solvents were obtained by distillation from suitable dessicants. The differents reagents were obtained commercially from Avocado or from Aldrich and were used without further purification. Analyses indicated by

[1] MGMT (O^6-methylguanine-DNA-methyltransferase); Mer^+ (methyl excision repair sufficient); Mer^- (methyl excision repair deficient).

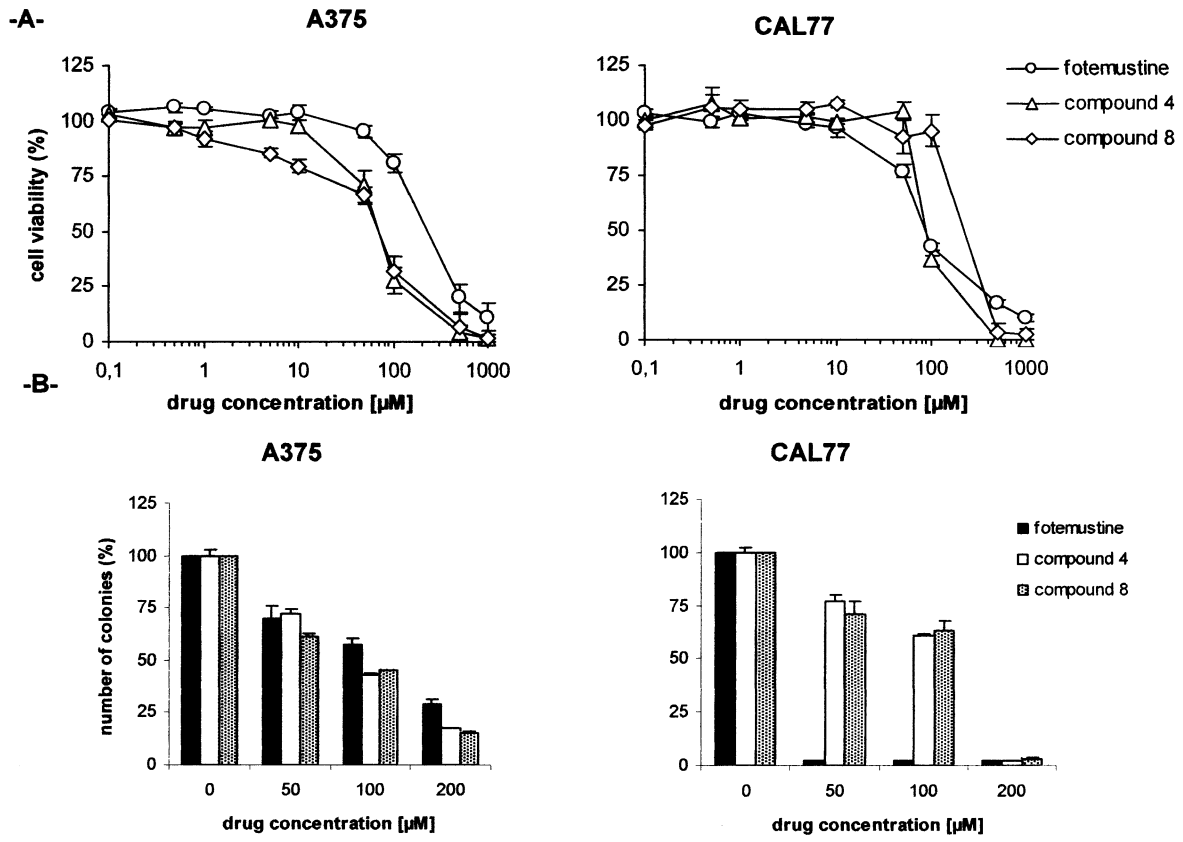


Fig. 3. Sensitivity of Melanoma cell lines to Fotemustine and analogues **4** and **8**. (A) Neutral red assay; (B) clonogenic assay.

symbols of the element were within ±0.4% of theorical values.

*5.1.1. O,O-diethyl-1-[[N′-(1′,1′-dimethylethoxy)carbonyl]sulfamido]ethanephosphonate (**2**)*

A solution of tertbutoxysulfamoyl chloride (prepared ab initio adding, chlorosulfonylisocyanate (1.1 equiv.) to a solution of *tert*-butanol (1.1 equiv.) in methylene chloride at 0 °C) was added to a solution of compound **1** (1 g, 1 equiv.) and triethylamine (1.15 ml, 1.5 equiv.) in 15 ml of methylene chloride at 0 °C. The mixture was then stirred at room temperature (r.t.) for 5 h, then diluted with methylene chloride. The mixture was then washed twice with water. The organic layer was dried over anhydrous sodium sulfate and concentrated in vaccuo. The residue was purified by chromatography on silica gel (eluent: ethylic ether) to give the expected compound as white solid. Yield: 52%; mp: 120–122 °C; R_f (ether) = 0.23; ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 5.63 (1H, d, J = 9.6 Hz), 4.18 (m, 6H), 1.48 (s, 9H), 1.35 (m, 9H); ^{31}P-NMR (250 MHz, CH_2Cl_2, DMSO-d^6 probe) δ: 24.4; ^{13}C-NMR (400 MHz, ^{1}H decoupled, $CDCl_3$) δ: 150.8, 83.9, 63.8 (d, J = 6.4 Hz), 63.3 (d, J = 6.4 Hz), 46.9 (d, J = 160 Hz), 28.4, 16.8 (d, J = 5.9 Hz), 16.7 (d, J = 5.9 Hz), 15.9, MS ESI$^+$ 30eV m/z: 383 $[M+Na]^+$. Anal. $C_{11}H_{25}N_2O_7PS$ (C, H, N).

*5.1.2. O,O-diethyl-1-[[N′-(2-chloroethyl)-N′-(1,1-dimethylethoxy)carbonyl]sulfamido]ethane phosphonate (**5**) and O,O-diethyl-1-[[N,N′-di-(2-chloroethyl)-N′-(1,1-dimethylethoxy) carbonyl]sulfamido]ethanephosphonates) (**3**)*

Diisopropylazodicarboxylate (0.549 ml, 1.5 equiv.) was added dropwise to a solution of compound **2** (0.665 g, 1 equiv.), triphenylphosphine (0.726 mg, 1.5 equiv.) and 2-chloroethanol (0.148 ml, 1.5 equiv.) in 6 ml of THF at 0 °C. The reaction mixture was stirred 4 h at r.t. and then concentrated under reduced pressure. The residue was purified by silica gel chromatography to afford compounds **5** and **3** in a 2/3–1/3 ratio. Compound **5**: yield: 40%; m.p.: 88–89 °C; R_f (ether) = 0.52; ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 5.7 (s, 1H), 4.15 (m, 5H), 3.95 (t, 2H, J = 6.36 Hz), 3.65 (t, 2H, J = 6.5 Hz), 1.53 (s, 9H), 1.44 (d, 3H, J = 7.2 Hz), 1.36 (d, 3H, J = 7.2 Hz), 1.32 (t, 6H, J = 7.2 Hz); ^{31}P-NMR (250 MHz, CH_2Cl_2, DMSO-d^6 probe) δ: 23.9; ^{13}C-NMR (400 MHz, ^{1}H decoupled, $CDCl_3$) δ: 151.8, 85.3, 63.8 (d, J = 6.5 Hz), 63.2 (d, J = 6.5 Hz), 48.8 (d, J = 160 Hz), 42, 28.4, 16.8 (d, J = 5.7 Hz), 16.7 (d, J = 5.7 Hz), 15.9; MS ESI$^+$ 30eV m/z: 445 $[M+Na]^+$. Anal. $C_{13}H_{28}ClN_2O_7PS$ (C, H, N). Compound **3**: yield: 20%; m.p.: 57–59 °C; R_f (ether) = 0.56; ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 4.15 (m, 5H), 3.95 (t, 2H, J = 7.2 Hz), 3.72 (m, 6H), 1.55 (s, 9H), 1.4 (2d, 3H, J = 7.4 Hz), 1.33 (2t,

3H, $J = 7$ Hz); ^{31}P-NMR (250 MHz, CH_2Cl_2, DMSO-d^6 probe) δ: 24.2; ^{13}C-NMR (400 MHz, ^{1}H decoupled, $CDCl_3$) δ: 151.2, 85.3, 63.2 (d, $J = 5.5$ Hz), 63.2 (d, $J = 5.5$ Hz), 50.62 (d, $J = 157$ Hz), 50.2, 47.4, 41.8, 41.3, 16.8 (d, $J = 3$ Hz), 16.7 (d, $J = 3$ Hz), 13.3 (d, $J = 2.7$ Hz); MS ESI$^+$ 30 eV m/z: 507 $[M+Na]^+$. Anal. $C_{15}H_{31}Cl_2N_2O_7PS$ (C, H, N).

*5.1.3. O,O-diethyl-1-[[N-methyl-N′-(2-chloroethyl)-N′-(1,1-dimethylethoxy)carbonyl]sulfamido]ethanephosphonate (**6**)*

Methyl iodide (0.03 ml, 2 equiv.) was added to a solution of compound **5** (0.117 g, 1 equiv.) and potassium carbonate (0.055 g, 1.5 equiv.) in acetone. The reaction mixture was stirred 3 h at r.t., then filtered over Celite. The filtrate was concentrated under reduced pressure and the resulting residue purified on silica gel (eluent: methylene chloride–acetone 9:1). Yield: 97%; m.p.: 68–70 °C; R_f (ether) = 0.52; ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 4.35 (2q, 1H, $J = 7.1$ Hz), 4.17 (m, 4H), 3.95 (t, 2H, $J = 6.6$ Hz), 3.67 (t, 2H, $J = 6.8$ Hz), 3.03 (s, 3H), 1.54 (s, 9H), 1.45 (2d, 3H, $J = 7.2$ Hz), 1.36 (2t, 6H, $J = 7$ Hz); ^{31}P-NMR (250 MHz, CH_2Cl_2, DMSO-d^6 probe) δ: 24.6; ^{13}C-NMR (400 MHz, ^{1}H decoupled, $CDCl_3$) δ: 151.4, 84.8, 63.4 (d, $J = 6.9$ Hz), 62.7 (d, $J = 6.9$ Hz), 50.7 (d, $J = 159$ Hz), 50, 41.9, 31.8, 16.8 (d, $J = 6$ Hz), 16.7 (d, $J = 6$ Hz), 12.4 (d, $J = 2.8$ Hz); MS ESI$^+$ 30eV m/z: 459 $[M+Na]^+$. Anal. $C_{14}H_{30}ClN_2O_7PS$ (C, H, N).

5.1.4. General procedure for Boc removal

N′-protected sulfamide was dissolved in a solution of 20% TFA in methylene chloride.The reaction mixture was stirred at room temperature until complete comsuption of starting material (TLC monitoring). After neutralization with a diluted aqueous solution of sodium hydrogenocarbonate and extraction with methylene chloride, the organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure.

5.1.5. General procedure for N-nitrosation

The sulfamide (1 equiv.) was dissolved in a mixture formic acid–methylene chloride 7:3, and sodium nitrite (6 equiv.) was added at 0 °C by small portions over 30 min. Then the mixture was neutralized with a diluted aqueous solution of sodium hydrogenocarbonate and extracted with methylene chloride. The organic layer was washed twice with water, dried over anhydrous sodium sulfate and concentrated in vaccuo to afford the nitrosated compound in quantitative yield.

*5.1.5.1. O,O-diethyl-1-[[N,N′-di-(2-chloroethyl)-N′-nitroso]sulfamido]ethanephosphonate (**4**).* R_f (ether) = 0.47; ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 4.5 (2q, 1H, $J = 7.2$ Hz), 4.15 (m, 4H), 3.8 (m, 6H), 3.5 (t, 2H, $J = 6.8$ Hz), 1.45 (2d, 3H, $J = 7.4$ Hz), 1.35 (2t, 6H, $J = 7$ Hz); ^{31}P-NMR (250 MHz, CH_2Cl_2, DMSO-d^6 probe) δ: 22.1; ^{13}C-NMR (400 MHz, ^{1}H decoupled, $CDCl_3$) δ: 63.6 (d, $J = 7.6$ Hz), 63.5 (d, $J = 7.6$ Hz), 51.5 (d, $J = 158$ Hz), 48, 41, 16.8 (d, $J = 3.8$ Hz), 16.7 (d, $J = 3.8$ Hz), 14; MS ESI$^+$ 30eV m/z: 437 $[M+Na]^+$.

*5.1.5.2. O,O-diethyl-1-[[N′-(2-chloroethyl)-N′-nitroso]sulfamido]ethanephosphonate (**7**).* R_f (ether) = 0.45; ^{31}P-NMR (250 MHz, CH_2Cl_2, DMSO-d^6 probe) δ: 24.2.

*5.1.5.3. O,O-diethyl-1-[[N-methyl-N′-(2-chloroethyl)-N′-nitroso]sulfamido]ethanephosphonate (**8**).* R_f (ether) = 0.45; ^{1}H-NMR (250 MHz, $CDCl_3$) δ: 4.41 (2q, 1H, $J = 7.2$ Hz), 4.15 (m, 6H), 3.51 (t, 2H, $J = 6.8$ Hz), 3.11 (s, 3H, $J = 6.8$ Hz), 1.41 (2d, 3H, $J = 7.4$ Hz), 1.35 (2t, 6H, $J = 7$ Hz); ^{31}P-NMR (250 MHz, CH_2Cl_2, DMSO-d^6 probe) δ: 23.2; ^{13}C-NMR (400 MHz, ^{1}H decoupled, $CDCl_3$) δ: 63.7 (d, $J = 7$ Hz), 63 (d, $J = 7$ Hz), 51.2 (d, $J = 159$ Hz), 44.4, 39.3, 31.2, 16.8 (d, $J = 5.7$ Hz), 16.7 (d, $J = 5.7$ Hz), 12.6; MS ESI$^+$ 30eV m/z: 388 $[M+Na]^+$.

Acknowledgements

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