Bioorganic & Medicinal Chemistry Letters 18 (2008) 4059–4063

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

N-(2,2-Dimethyl-2-(2-nitrophenyl)acetyl)-4-aminocyclophosphamide as a potential bioreductively activated prodrug of phosphoramide mustard

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ARTICLE INFO

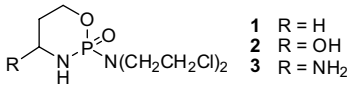
Article history:
Received 2 May 2008
Accepted 27 May 2008
Available online 13 June 2008

Keywords:
Prodrug
Cyclophosphamide
Phosphoramide mustard
Bioreductive activation
Cyclization

ABSTRACT

N-(2,2-Dimethyl-2-(2-nitrophenyl)acetyl)-4-aminocyclophosphamide isomers (DMNA-NH-CPA, **4**) were synthesized stereospecifically from Boc-L-Hse(OBn)-OH and the degradation of the corresponding reduced amine **5a** was investigated by UV/vis spectroscopy and LC/MS. The rate of cyclization of **5a** was found to increase with decreasing pH, with half-lives ranging from 3.2 to 54 min at pH 4–7.4, suggesting that the cyclization is catalyzed by the hydronium ions. LC/MS analysis of the degradation products of **5a** indicates that 4-aminocyclophosphamide is rapidly released from **4** upon reductive activation under acidic conditions and further decomposes into the cytotoxic phosphoramide mustard. These results validated 4-aminocyclophosphamide as a prodrug form of phosphoramide mustard and suggest that compound **4** can potentially be used as a prodrug of phosphoramide mustard for bioreductive activation.

Hypoxia is a unique physiological characteristic of solid tumors and is, therefore, an important therapeutic target.[1] The oxygen deficiency of solid tumors often leads to resistance to ionizing radiation and to many chemotherapeutic drugs. On the other hand, it also provides an opportunity for selective anticancer chemotherapy. Hypoxic tumor cells are known to have a greater capacity for reductive reactions as compared to well-oxygenated normal cells. The difference has been used to explore bioreductively activated prodrugs for higher therapeutic index, some of which are currently in clinical trials.[2,3] Cyclophosphamide (**1**) is an alkylating antitumor agent with activity against a broad spectrum of human cancers including slow-growing tumors.[4] However, the clinical application of cyclophosphamide is limited due to its dose-related toxic side effects, including the life-threatening hemorrhagic cystitis. Consequently, there has been considerable attention in designing prodrugs that specifically release cytotoxic phosphoramide mustard at tumor sites, thus minimizing toxic side effects.[5–7] We have recently reported for the first time that 4-aminocyclophosphamide (4-NH_2-CPA, **3**) can be used as a prodrug form of phosphoramide mustard because of its structural similarity to 4-hydroxycyclophosphamide (**2**) and its spontaneous degradation as a mono-phosphorylated *gem*-diamine.[8] 4-Aminocyclophosphamide can be easily incorporated into a prodrug through a stable amide bond, for example, in peptide conjugates for proteolytic activation. In this communication, we report the synthesis of a pair of *N*-(2,2-dimethyl-2-(2-nitrophenyl)acetyl)-4-aminocyclophosphamide diastereomers (**4**) and the degradation of the corresponding reduced amine intermediate in buffer. Our results suggest that compound **4** could potentially be used as a prodrug of phosphoramide mustard for bioreductive activation in selective cancer chemotherapy.



1 R = H
2 R = OH
3 R = NH_2

N-(2,2-Dimethyl-2-(2-nitrophenyl)acetyl)-4-aminocyclophosphamide (**4**) was designed to release 4-aminocyclophosphamide (**3**) through a bioreductive activation mechanism involving reduction and a spontaneous cyclization–elimination reaction (Scheme 1). Nitroaryl amides are stable compounds; however, their reduced products, the corresponding amine or hydroxylamine derivatives, are prone to undergo an intramolecular cyclization–elimination reaction.[9] This feature has been widely explored to develop prodrugs for bioreductive activation.[10] Because of the conformational restriction by the two methyl groups, the reduced amine or hydroxylamine intermediates of the 2,2-dimethyl-2-(2-nitrophenyl)acetyl derivatives are known to cyclize much faster than the corresponding analogs without the two methyl substitu-

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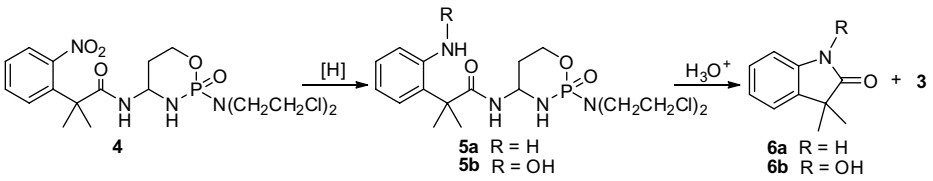
doi:10.1016/j.bmcl.2008.05.099



Scheme 1. Proposed mechanism of reductive activation of *N*-(2,2-dimethyl-2-(2-nitrophenyl)acetyl)-4-aminocyclophosphamide (**4**).

ents.[9,11] Our previous work demonstrated that the 2,2-dimethyl-2-(2-nitrophenyl)acetate of FUDR cyclized within seconds upon reduction.[12] The 2,2-dimethyl-2-(2-aminophenyl)acetamides were found to be stable under neutral hydrogenation conditions at room temperature but quickly cyclize upon treatment with acid. The fast release of 4-aminocyclophosphamide (**3**) from compound **4** also provided a system to investigate the degradation pathways of **3**.

Similar to the phenylalanyl 4-aminocyclophosphamides,[8,14] compound **4** has four configurational stereoisomers which can be referred to as (2*R*,4*R*)-, (2*R*,4*S*)-, (2*S*,4*R*)-, and (2*S*,4*S*)-**4**. As the configuration of 4-aminocyclophosphamide was expected to have little effect on the cyclization reaction of **5a**, only one pair of diastereomers of **4**, (2*S*,4*S*)- and (2*R*,4*S*)-, were synthesized stereospecifically from a protected L-homoserine and the degradation of their corresponding amine intermediates was investigated. As shown in Scheme 2, the synthesis of (2*S*,4*S*)- and (2*R*,4*S*)-**4** started from Boc-L-Hse(Bn)-OH (**7**) which was first converted into its amide **8** by HOBt/EDC activation followed by treatment with saturated ammonium hydroxide. After removal of the Boc group in **8**, the free amino group was allowed to react with 2,2-dimethyl-2-(2-nitrophenyl)acetyl chloride (DMNA-Cl) to give **10**. DMNA-Cl was prepared by refluxing the corresponding acid in $SOCl_2$. The bis(trifluoroacetoxy)iodobenzene (BTI)-mediated Hoffmann rearrangement was employed to convert the amide **10** to the corresponding monoacylated *gem*-diamine derivative **11**. The benzyl group in **11** was removed by treatment with BBr_3 in CH_2Cl_2 at −50 to 0 °C under argon to give 1,3-aminoalcohol **12**. Lactam **6a** was isolated as the major side product from the degradation of *gem*-diamines **11** and **12**.[15] Attempt to remove the benzyl protecting group using trimethylsilyl iodide was not successful.[16] Cyclization of **12** with bis(2-chloroethyl)phosphoramidic dichloride afforded a mixture of the (2*S*,4*S*)- and (2*R*,4*S*)-diastereomers of **4**, which were easily separated by flash column chromatography, yielding a faster-eluting cis-diastereomer ((2*S*,4*S*)-**4**) and a slower-eluting trans-diastereomer ((2*R*,4*S*)-**4**).[17] Assignment of the (2*S*,4*S*)- and (2*R*,4*S*)-configurations was also based on their ^1H and ^{31}P NMR shifts and has been extensively discussed previously.[8,14]

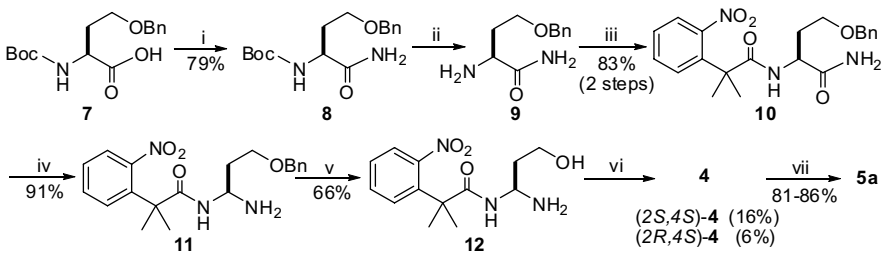
The stability of the (2*S*,4*S*)- and (2*R*,4*S*)-diastereomers of **4** under physiological conditions was examined by incubating the isomers at pH 7.4 (phosphate buffer) and 37 °C. HPLC analysis of the incubation mixtures showed no significant changes over a period of three days (data not shown). To investigate the mechanism of reductive activation of compound **4**, the corresponding reduced amine product **5a** was synthesized by hydrogenation over 5% Pd–C in methanol at room temperature for 30 min. The hydrogenation reaction was easily monitored by TLC. Crude product solutions of 2-nitroaryl amides upon reduction were previously used to investigate such cyclization reactions.[9] However, we found that partial cyclization of the amine intermediates of **4** could not be avoided during the hydrogenation process; but, **5a** was sufficiently stable to be purified by flash column chromatography on silica gel. Similar results were observed for the 2,2-dimethyl-2-(*o*-nitrophenyl)acetamides of amino acids.[13] The isolated yields of **5a** were 81–86%. Hydrogenation using platinum catalyst such as PtO_2 was accompanied with significant cyclization reactions, with both lactam **6a** and hydroxylactam **6b** isolated as side products. This is consistent with the observation that platinum-catalyzed hydrogenation generates more hydroxylamine than the amine product[13] and the hydroxylamine product cyclizes much faster than the amine product because of its higher nucleophilicity.[18]

To examine the pH-dependence of the cyclization reaction, (2*S*,4*S*)-**5a** was incubated at 37 °C in a series of buffers with pH

Table 1
Cyclization rate constants of (2*S*,4*S*)-**5a** under varying pH conditions at 37 °C

pH[a]	k_{obs}[b] (min^{-1})	$t_{1/2}$[c] (min)
4.0	0.22	3.2
4.5	0.14	4.9
5.0	0.071	9.8
5.4	0.04	17.5
5.6	0.025	27.2
6.0	0.055	12.6
6.5	0.036	19.3
7.4	0.013	53.7
8.0	—[d]	—[d]

[a] Buffer components were 100 mM sodium acetate for pH 4.0–5.6, 100 mM sodium phosphate for pH 6.0–7.4, 100 mM Tris/HCl for pH 8.0.
[b] k_{obs} = observed pseudo-first-order rate constants for cyclization reaction. Substrate (10 μM) was incubated in the desired buffer at 37 °C and changes of UV absorbance at 249 nm were recorded for the calculation of k_{obs}.
[c] $t_{1/2}$ (half-life of **5a**) = 0.693/k_{obs}.
[d] No cyclization was observed for 3 h.



Scheme 2. Synthesis of *N*-(2,2-dimethyl-2-(2-nitrophenyl)acetyl)-4-aminocyclophosphamide (**4**). Reagents and conditions: (i) HOBt, EDC, THF, rt, then sat'd NH_3 (aq); (ii) TFA/CH_2Cl_2 (1/3), rt, 1 h; (iii) DMNA-Cl, CH_2Cl_2, rt, 5 h; (iv) BTI, CH_3CN/H_2O (1/1), rt, 4 h; (v) BBr_3, THF, −50 to 0 °C, 1.5 h; (vi) $Cl_2PON(CH_2CH_2Cl)_2$, TEA, THF, 0 °C–rt, 48 h; (vii) H_2, 5% Pd–C, MeOH, rt, 30 min.

ranging from 4 to 8 and the rate of cyclization was monitored by measuring the UV absorbance change at 249 nm corresponding to the maximum absorption wavelength of the cyclized product **6a**.[12] Table 1 lists the observed pseudo first order rate constants of cyclization and the half-lives of compound (2*S*,4*S*)-**5a** under different pH conditions. Kinetics of cyclization of similar systems have been studied previously by others.[9] Consistent with results reported by others, the cyclization rate constant of (2*S*,4*S*)-**5a** was found to be proportional to hydronium ion concentration as indicated by the plot of log(k_{obs}) versus pH shown in Figure 1. The slight jump in the observed pseudo first order rate constants in the pH range between 5.6 and 6 was attributed to the change in the buffer ion species from acetate to phosphate.[9] The shortest half-life was 3.2 min at pH 4 and the longest was 53.7 min at pH 7.4 (Table 1). No significant cyclization was found at or above pH 8.0. These results suggest that the cyclization reaction is catalyzed by the hydronium ions. Given that the solid tumor cells are often associated with an acidic microenvironment (pH ~ 6),[19] half-lives of (2*S*,4*S*)-**5a** at pH 6.0 and 7.4 indicates that the cyclization of **5a** in the solid tumors could be more than four-fold faster than that under normal physiological pH conditions.

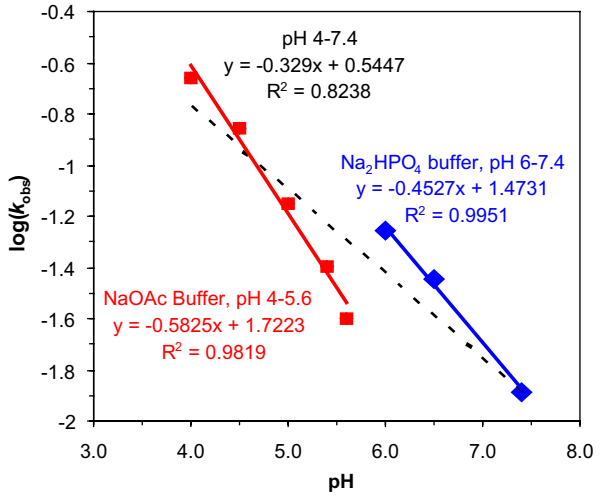


Figure 1. pH-dependence of the observed pseudo first order rate constants of cyclization of (2*S*,4*S*)-**5a** at 37 °C.

To identify the products of cyclization, compound (2*S*,4*S*)-**5a** was incubated in 100 mM phosphate buffer at pH 6 and 37 °C and the reaction progress was monitored by LC/MS at different time intervals. The incubation conditions were chosen based on the short half-life (12.6 min) of (2*S*,4*S*)-**5a** under these conditions and their similarity to the acidic microenvironment of solid tumor cells (pH ~ 6).[19] LC/MS analysis indicated that the cyclization reaction was nearly complete within 3 h as indicated by the disappearance of (2*S*,4*S*)-**5a** and two product peaks were observed based on the UV trace (220 nm) (Fig. 2A). The major product peak (>90%) was identified as the lactam **6a** by the corresponding mass spectrum and comparison with the authentic sample. The minor product peak (<10%) showed a molecular ion (MH^+) of 161, one unit less than that of the lactam, and was assigned as 3,3-dimethyl-3*H*-indol-2-ylamine (**15**) as shown in Scheme 3 (path A). Formation of **15** was attributed to be the decomposition of the intermediate **13** which could be formed through the cyclization–dehydration (path A) of (2*S*,4*S*)-**5a**. Presence of **13** in the reaction mixture was confirmed by the selected ion extraction of its molecular ion (419) and the observation of the corresponding characteristic isotopic peaks of two chlorines (Fig. 2B, inset). LC/MS analysis of the incubation mixtures at different time intervals indicated that compound **13** was formed as a minor component in the incubation mixture and decreased with the increasing formation of **15**. Formation of **15** from **13** suggested the release of cyclophosphamide-3-ene (**14**) as the other product. While **14** was not directly detected by LC/MS, formation of **14** in the incubation mixture was confirmed by the detection of its cyanide and thiolate adducts in the presence of the corresponding trapping reagents (see below). Attempts to amplify the formation of **13** through path A from

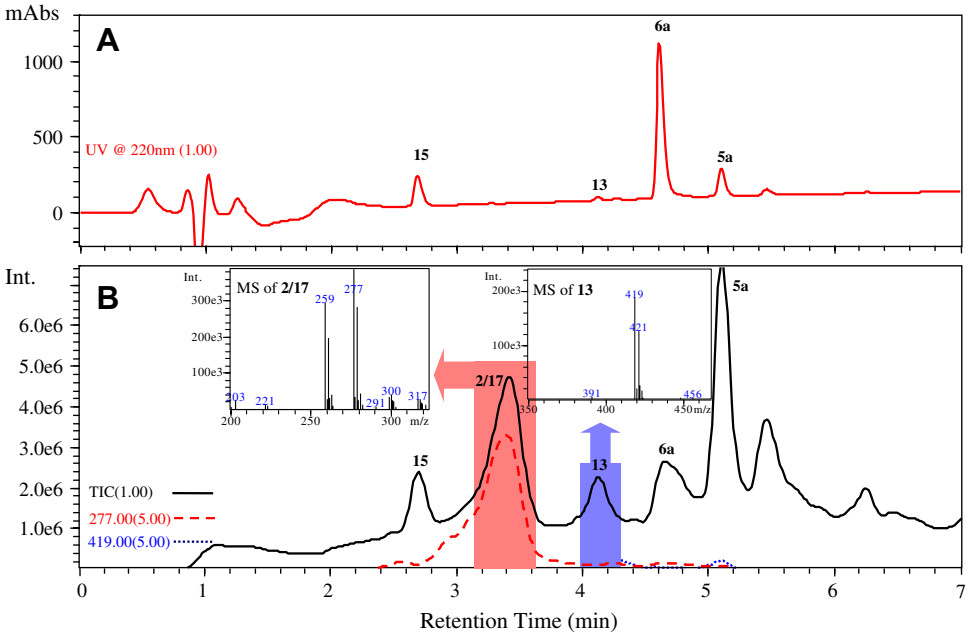
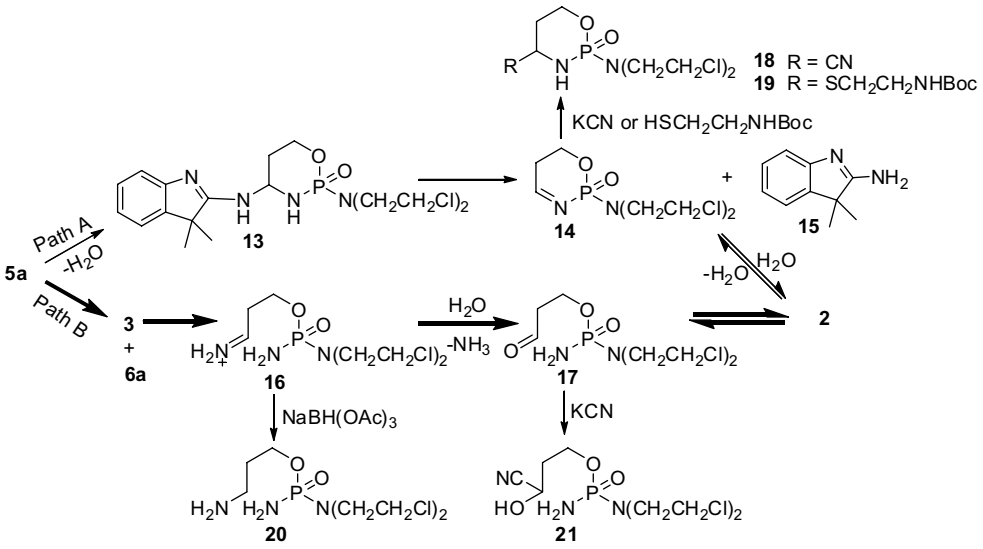


Figure 2. LC/MS chromatogram of the incubation mixture of (2*S*,4*S*)-**5a** in 100 mM phosphate buffer at pH 6 and 37 °C for 3 h: (A) UV trace (220 nm) showing peaks corresponding to amidine **15**, intermediate **13**, lactam **6a**, and starting amine **5a**; (B) total ion chromatogram (TIC) showing peaks corresponding to amidine **15**, 4-hydroxylcyclophosphamide (**2**)/aldophosphamide **17**, intermediate **13**, lactam **6a**, and starting amine (2*S*,4*S*)-**5a**. Selected ion chromatograms of **2/17** (----) and **13** (·····) are also shown with their corresponding MS spectra as inserts.



Scheme 3. Proposed mechanisms of degradation of compound (2*S*,4*S*)-**5a** at pH 6 and 37 °C.

(2*S*,4*S*)-**5a** using dehydration reagents were not successful,[20] indicating that the dehydration pathway is not favored in comparison to the alternative elimination pathway and the dehydrated product **13** is not stable.

Observation of >90% lactam in the incubation mixture suggested that compound (2*S*,4*S*)-**5a** mainly went through the cyclization–elimination pathway and 4-aminocyclophosphamide (**3**) was released (path B). However, attempts to detect compound **3** in the incubation mixtures at different time intervals by LC/MS were not successful. Instead, 4-hydroxycyclophosphamide (**2**) or aldophosphamide **17** was unambiguously identified in these mixtures by the presence of the correct molecular ion of 277 and its major fragmentation peak 259 (MH^+–H_2O), both with their characteristic isotopic peaks corresponding to the presence of two chlorines (Fig. 2B and MS inset). Other potential degradation products of 4-aminocyclophosphamide were also examined but not found, including iminophosphamide **16**, cyclophosphamide-3-ene (**14**), and phosphoramide mustard. To trap these short-lived reactive intermediates during the decomposition of 4-aminocyclophosphamide, cyclization of (2*S*,4*S*)-**5a** was carried out under various conditions in the presence of different trapping reagents and the corresponding products were analyzed by LC/MS. The reduced amine product **20** from the imine intermediate **16** was detected when reduction of (2*S*,4*S*)-**4** was carried out in the presence of 10% acetic acid by hydrogenation or incubation of (2*S*,4*S*)-**5a** in a buffer containing sodium borohydride acetate. Imines are known to be reduced to amines under these conditions.[21,22] The amine product was able to be further trapped by a preformed HOBt ester of Z-Tyr(OBn)-OH. Incubation of (2*S*,4*S*)-**5a** at 37 °C in phosphate buffer (pH 6.0) containing KCN or Boc–NH–CH_2–CH_2–SH also gave the corresponding cyanide or thiolate adducts of **14** and **17**, respectively, as shown in Scheme 3.

The above results clearly suggest that 4-aminocyclophosphamide was released from compound (2*S*,4*S*)-**5a** upon cyclization and further decomposed into the cytotoxic phosphoramide mustard. Based on the intermediates detected, the degradation mechanism of (2*S*,4*S*)-**5a** was proposed as shown in Scheme 3. Compound (2*S*,4*S*)-**5a** decomposes under acidic conditions mainly through a cyclization–elimination path B to release 4-aminocyclophosphamide, accompanied by a minor cyclization–dehydration path A to form intermediate **13**. Both degradation pathways would lead to the formation of 4-hydroxycyclophosphamide (**2**) which is responsible for the formation of the ultimate alkylating agent phosphoramide mustard, the active metabolite of cyclophosphamide.[23] Detection of significant formation of **15** indicates that **13** disintegrates into **14** which is known to be in equilibrium with **2**. Formation of **2** in the medium is also supported by its trapping as cyanide and thiolate adducts. Although detection of **14** and **2** would also support a NH_3-elimination pathway of 4-aminocyclophosphamide (**3**), this route was ruled out for the degradation of *gem*-diamines in a previous mechanistic study.[15] Instead, detection of **16** and **17** as trapped intermediates suggests that 4-aminocyclophosphamide disintegrates by the expulsion of the phosphoramide, similar to the hydrolysis of its acyl amide counterparts. The aldophosphamide **17** is known to be in equilibrium with **2** and disintegrate spontaneously into acrolein and phosphoramide mustard through a base-catalyzed β-elimination.[23]

In summary, *N*-(2,2-dimethyl-2-(2-nitrophenyl)acetyl)-4-amino cyclophosphamide (**4**) was synthesized as a potential prodrug of phosphoramide mustard for reductive activation. Compound **4** is stable under physiological conditions but rapidly releases 4-aminocyclophosphamide after reduction of the nitro group to hydroxylamine and/or amine under acidic conditions. The cyclization of the reduced amine intermediate **5a** is catalyzed by acid. LC/MS analysis of the decomposition products of (2*S*,4*S*)-**5a** suggests that 4-aminocyclophosphamide disintegrates into an iminophosphamide which is further hydrolyzed to aldophosphamide to release the alkylating agent phosphoramide mustard. These results clearly demonstrate that 4-aminocyclophosphamide is an effective prodrug form of phosphoramide mustard and compound **4** could potentially be used as a prodrug for bioreductive activation to improve the therapeutic effectiveness of cyclophosphamide in the treatment of cancer.

Acknowledgements

We gratefully acknowledge the financial support of Grant SNJ-CCR 700-009 from the State of New Jersey Commission on Cancer Research, a pilot grant from the Gallo Prostate Cancer Center of the Cancer Institute of New Jersey, and Grant RSG-03-004-01-CDD from the American Cancer Society.

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17. (2*S*,4*S*)-**4**: R_f = 0.35 (CH_2Cl_2:CH_3OH, 20/1); ^{1}H NMR (200 MHz, CD_3OD) δ 7.87–7.46 (m, 4H), 5.31 (dt, 1H, *J* = 20.4 Hz), 4.60–4.50 (m, 1H), 4.31–4.20 (m, 1H), 3.70–3.63 (m, 4H), 3.50–3.36 (m, 4H), 2.20–2.00 (m, 2H), 1.74 (s, 3H), 1.70 (s, 3H); ^{13}C NMR (50 MHz, CD_3OD) 175.8, 148.8, 138.4, 132.4, 128.2, 127.3, 124.5, 63.5 (d, $^2J_{C,P}$ = 25.8 Hz), 59.0, 52.9, 48.4 (d, $^2J_{C,P}$ = 9.2 Hz), 40.9, 29.0 (d, $^3J_{C,P}$ = 26.4 Hz), 26.4, 25.8; ^{31}P NMR (121 MHz, CD_3OD) δ 12.4 (s); IR (film) 3351.3, 2975.8, 1667.8, 1529.8, 1359.9, 1231.0, 1059.1 cm^{-1}; LC/MS (ESI) 467.1 [MH]$^+$, 469.1; HRMS (FAB) *m*/*z* calcd for 467.1018, found 467.1030, 469.1001. (2*R*,4*S*)-**4**: R_f = 0.20 (CH_2Cl_2:CH_3OH, 20/1); ^{1}H NMR (200 MHz, CD_3OD) δ 7.88–7.47 (m, 4H), 5.38 (dt, *J* = 6.9, 2.6 Hz, 1H), 4.35–4.24 (m, 2H), 3.71–3.63 (m, 4H), 3.52–3.37 (m, 4H), 2.03–1.97 (m, 2H), 1.70 (s, 6H); ^{13}C NMR (200 MHz, CD_3OD) δ 176.3, 148.9, 138.3, 132.5, 128.2, 127.4, 124.6, 63.8, 58.8, 41.1, 29.3 (d, $^3J_{C,P}$ = 14 Hz), 26.3; ^{31}P NMR (121 MHz, CD_3OD) δ 14.1 (s); IR (film) 3272.5, 2928.0, 1704.2, 1667.9, 1504.9, 1455.4, 1367.4, 1216.6, 1168.6, 1048.9, 988.5 cm^{-1}; LC/MS (ESI) 467.1 [MH]$^+$, 469.1; HRMS (FAB) *m*/*z* calcd for 467.1018, found 467.1042.
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