Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 1 June 2009, Revised 8 August 2009, Accepted 11 August 2009 Published online 21 October 2009 in Wiley Interscience

(www.interscience.wiley.com) DOI: 10.1002/JLCR.1676

Carbon-14 radiosynthesis of combretastatin A-1 (CA1) and its corresponding phosphate prodrug (CA1P)

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The natural product combretastatin A-1 (CA1) is isolated from the African bush willow tree, a member of the *Combretaceae* family. CA1 has important medicinal value, due in part to its ability to inhibit tubulin assembly. The prodrug combretastatin A-1 diphosphate (CA1P; OXi4503) is currently in human Phase I clinical trials as a vascular disrupting agent. This paper describes the carbon-14 radiosynthesis of [4′-^{14}C]CA1 and the corresponding phosphate prodrug salt [4′-^{14}C]CA1P in high specific activity (55 mCi/mmol). The carbon-14 label was introduced by methylation of the C-4′ protected phenolic moiety of the CA1 precursor following removal of the *tert*-butyldimethylsilyl protecting group in the presence of [^{14}C]methyl iodide. This was accomplished in excellent yield without significant *Z* to *E* isomerization. The [^{14}C]-precursor ((*Z*)-1-[3′,[4′-^{14}C],5′-trimethoxyphenyl]-2-[2″,3″-di-[(*iso*propyl)oxy]-4″-methoxyphenyl] ethene) was subjected to a de-*iso*propylation reaction with $TiCl_4$. The tetrabenzyl phosphate derivative of the resulting diol was prepared using fresh dibenzyl phosphite. Debenzylation with trimethylsilylbromide, followed by hydrolysis of the trimethylsilyl ester and adjustment of the pH with dilute aqueous hydrochloric acid yielded [4′-^{14}C]CA1P with an overall radiochemical yield of 8.4%.

Keywords: tumour; combretastatin; OXi4503; CA1P; CA1; CA4

Introduction

The hydroxyl stilbenoid natural products combretastatin A-1 (CA1) and combretastatin A-4 (CA4) (Figure 1) are microtubule depolymerization agents. They are members of a family of 17 compounds, first isolated from the bark of the African bush willow tree *Combretum caffrum* by George R. Pettit and co-workers in the early 1980s.[1]

OXiGENE Inc. (Waltham, MA) is currently developing the phosphate ester prodrugs combretastatin A-1 diphosphate (CA1P; OXi4503) and combretastatin A-4 phosphate (CA4P; Zybrestat™, fosbretabulin) for the treatment of advanced solid tumors.[2]

CA1P and CA4P are classed as vascular disrupting agents (VDAs)[3] and have associated cancer therapy properties. These compounds show selective activity against the vasculature of cancerous tumors.

Biological mode of action

In the case of CA1P and CA4P, the mode of action has been shown to be initial dephosphorylation, followed by binding of the free combretastatin to the tubulin heterodimer. This results in an irreversible process of microtubule depolymerization, and a cascade of cell signaling events. In normal blood vessels, this tubulin binding has little effect because these vessels also have a well-organized 'scaffolding' system to keep them intact. However, in the microvessels feeding abnormal cells (tumors), or on the retina in some eye diseases, such supports are not present and the VDA causes the endothelial cell to become spherical. As these cells build up, they block the flow of blood, leading to the tumor and kill it by starving it of oxygen.[4]

In contrast to CA4P, OXi4503 also possesses direct cytotoxic activity. Preclinical studies have demonstrated that the additional phenolic moiety in CA1 as compared to A-4 markedly changes the redox properties of the molecule and introduces a completely different chemical functionality.[5] The oxidative enzymes (tyrosinases and peroxidases), which are present at elevated levels in various solid and liquid tumors, cause OXi4503 to undergo metabolic *in vivo* activation. This oxidative metabolism process results in the creation of reactive 'radical' oxygen species and an *ortho*quinone metabolite, which covalently binds to proteins and the nucleic acids in DNA, producing direct cytotoxic effects, leading eventually to tumor necrosis.[2]

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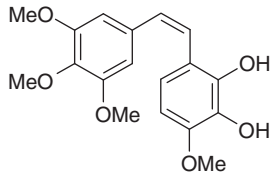
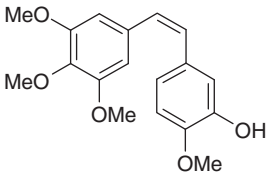
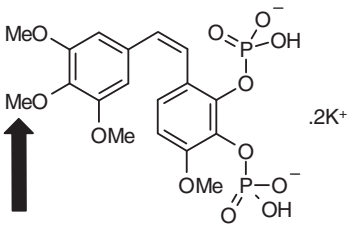


CA1



CA4

Figure 1. Combretastatins CA1 and CA4.



site of carbon-14 label

Figure 2. Position of carbon-14 label in CA1P.

In July 2007, the biotechnology company OXiGENE initiated a 180-patient phase III clinical trial of Zybrestat in combination with carboplatin for the treatment of anaplastic thyroid cancer (Study of Combretastatin and Paclitaxel/Carboplatin in the Treatment of Anaplastic Thyroid Cancer). There is currently no fully FDA approved treatment for this form of cancer.[2] OXi4503 (CA1P) is currently being evaluated as a monotherapy in a Phase 1 dose-escalation clinical trial in patients with advanced solid tumors.[6a]

Carbon-14 radiosynthesis

To elucidate the mechanism of metabolism of CA1P in ADME-toxicology studies it was important to incorporate a radio-isotope into the structure. Carbon-14 was chosen as the radiotracer due to its long half-life period (~5730 years) and the fact that it could be introduced without changing the chemical structure. The ease of replacement of the methoxy group at the 4′-position in the substrate 3′,4′,5′-trimethoxy-phenyl (Figure 2) made it a feasible location to incorporate a carbon-14 labelled methoxy moiety, although similar trimethoxyphenyl VDA compounds have been shown to undergo metabolic demethylation.[6b] [^{14}C]Methyl iodide was used as the methylating agent.

This paper describes the synthesis of [^{14}C]CA1P, using the route shown in Scheme 1. The synthetic methodology was based on the unlabelled route established by Pinney and co-workers at Baylor University.[7–9]

The orthogonally protected stilbene starting material (*Z*)-1-[3′,5′-dimethoxy-4′-[(*tert*-butyldimethylsilyl)oxy]phenyl]-2-[2″,3″-di-[(*iso*propyl)oxy]-4″-methoxyphenyl] ethene (**1**) was reacted with the fluorinating reagent TBAF and one equivalent of [^{14}C]methyl iodide, selectively introducing the carbon-14 label at the C4′ phenolic position. The original unlabelled synthesis used four equivalents of methyl iodide, but in this case, to avoid excess radiochemical waste and a low radiochemical yield, the number of equivalents was reduced to one. As there was no excess of [^{14}C]methyl iodide, it was important that the reaction solution was completely anhydrous to avoid protonation at C4′. This was achieved by the addition of activated 3 Å molecular sieves. Subsequently, both isopropyl groups of (*Z*)-1-[3′,[4′-^{14}C],5′-trimethoxyphenyl]-2-[2″,3″-di-[(*iso*propyl)oxy]-4″-methoxyphenyl] ethene (**2**) were removed using the Lewis acid titanium tetrachloride in dichloromethane, under carefully controlled temperature conditions. This deprotection procedure gave variable yields, particularly on a small scale. Utilizing a freshly opened, highly pure (99.999%) bottle of titanium tetrachloride, the reaction was completed within 40 min, although [^{14}C]CA1 (**3**) was obtained in lower yield than during the cold synthesis (27% cf. 45%).[9] The [^{14}C]CA1 obtained after the deprotection of the *iso*propyl groups was purified using silica gel capped with a plug of Florisil®, to remove potential titanium impurities. The removal of these impurities avoids possible isomerization during the salt formation step. After chromatography, the resultant yellow oil was recrystallized giving tan colored crystals of [^{14}C]CA1 (**3**), of good radiochemical purity by radio-TLC and ^{1}H NMR. The treatment of deprotected combretastatin (**3**) with dibenzyl chlorophosphate, generated *in situ* from dibenzyl phosphite and carbon tetrachloride, gave the tetrabenzyl protected phosphate product (**4**). The commercially available dibenzyl phosphite did not perform well in this step, thus the reagent was prepared fresh for use in the radiosynthesis.[10] This method of phosphorylation is a higher yielding alternative to the published method using *N*-chlorosuccinimide,[8] although it has the drawback of utilizing toxic carbon tetrachloride.

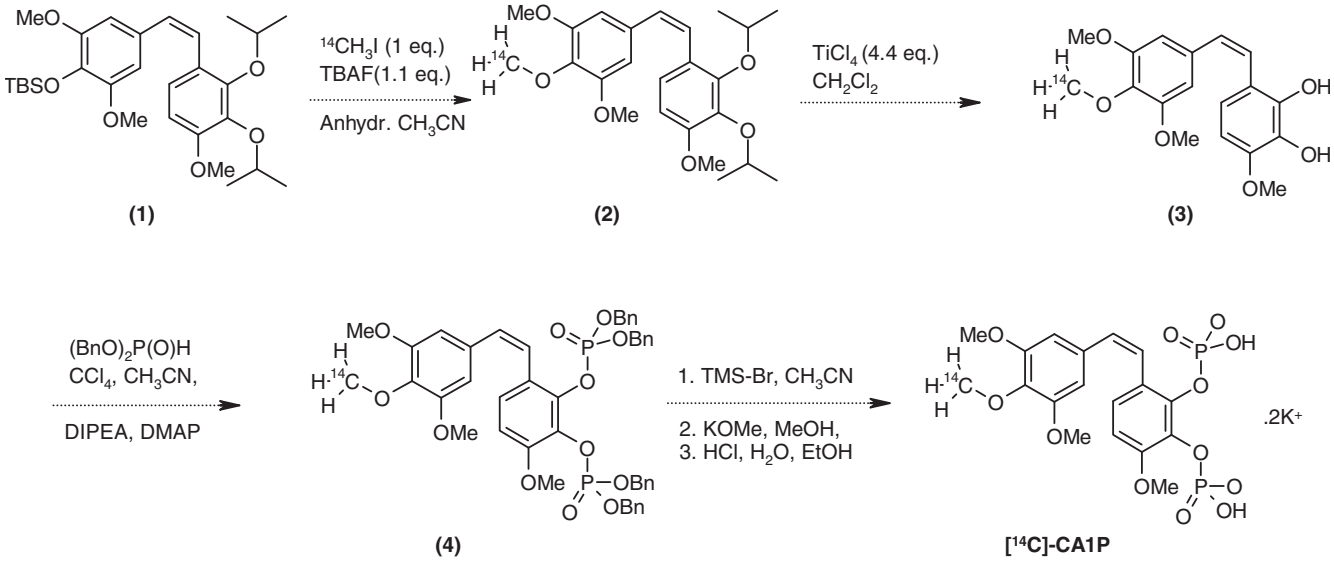
Finally, debenzylation of stilbene (**4**) using a solution of trimethylsilyl bromide in acetonitrile resulted in the *tetra*-trimethylsilyl phosphate ester, which was quenched into a solution of excess potassium methoxide in methanol to afford the *tetra* potassium salt along with other mono-, di-, and tri-potassium substituted salts. The mixture of salts was then dissolved in water, and the resultant aqueous solution was carefully adjusted to a pH between 4.75 and 4.85 using dilute hydrochloric acid, followed by precipitation of the di-potassium salt [^{14}C]CA1P with ethanol. The final product was characterized by ^{1}H NMR, analytical HPLC, and specific activity. [^{14}C]CA1P was obtained in an overall yield of 8.4%, with 97.5% chemical purity, 96.7% radiochemical purity by analytical HPLC area %, and with a specific activity of 55 mCi/mmol.

Conclusion

The carbon-14 labelled compound CA1P was prepared in four steps following the route developed by the Pinney group at Baylor University. The material was prepared in a radiochemical purity of greater than 96% by HPLC area % and an overall radiochemical yield of 8.4%.

Experimental

The reaction steps were performed under inert atmosphere using nitrogen gas unless specified differently. Chemical reagents used in the synthetic procedures were obtained from various chemical suppliers. [^{14}C]Methyl iodide was purchased from Perkin Elmer (>97%, 40–60 mCi/mmol). Anhydrous acetonitrile (99.8%) and titanium tetrachloride (99.999%) were purchased from Aldrich and used without further purification. A fresh bottle of titanium tetrachloride was used for each deprotection step. Anhydrous dichloromethane was freshly distilled from P_2O_5 followed by distillation from calcium hydride



Scheme 1. Synthesis of [^{14}C]-CA1P.

and storage over 3 Å molecular sieves. Trimethylsilyl bromide (98%, Aldrich) was freshly distilled from calcium hydride. Silica gel (200–400 mesh, 60 Å) was used for column chromatography. TLC plates (precoated glass plates with silica gel 60 F254, 0.25 mm thickness) were used to monitor reactions. Intermediates and products synthesized were characterized based on ^{1}H NMR (Bruker Avance operating at 500 MHz). All the chemical shifts are expressed in ppm (δ), coupling constants (*J*, Hz), and peak patterns are reported as broad (br), singlet (s), doublet (d), triplet (t), quartet (q), septet (sep), and multiplet (m). Purity of the final compound was further analyzed using an Agilent 1100 HPLC system with UV- and radiochemical detection; a Supelco Discovery C18 HPLC column 12.5 cm × 4.6 mm, 5 μm; $T = 25^\circ$C; eluants, solvent A, 25 mM tetrabutylammonium bromide (TBAB) with 0.1% trifluoroacetic acid (TFA) in water, solvent B, 25 mM TBAB with 0.08% TFA in water/acetonitrile (2/8 v/v); gradient, 80% A/20% B to 5% A/95% B over 0–45 min; flow rate, 0.7 mL/min; injection volume 25 μL; monitored at 264 nm wavelength.

Synthesis of (*Z*)-1-[3′,[4′-^{14}C], 5′-trimethoxyphenyl]-2-[2″,3″-di-[(*iso*propyl)oxy]-4″-methoxyphenyl] ethene (2)

This reaction was performed in two batches (total activity of 490 mCi of [^{14}C]methyl iodide with a specific activity range of 40–60 mCi/mmol). For each batch, (*Z*)-1-[3′,5′-dimethoxy-4′-[(*tert*-butyldimethylsilyl)oxy]phenyl]-2-[2″,3″-di-[(*iso*propyl)oxy]-4″-methoxyphenyl] ethene (**1**) (2.22 g; 4.30 mmol; 1.0 eq.) was dissolved in anhydrous acetonitrile (32 mL) and dried over activated 3 Å molecular sieves (9.5 g). [^{14}C]Methyl iodide (0.62 g; 4.30 mmol; 245 mCi; 1.0 eq.) was transferred to the flask using a manifold line and stirred for 20 min. TBAF, 1.0 M in THF (4.73 mL; 4.73 mmol; 1.1 eq.), was added and the reaction stirred at 0°C for 30 min. The reaction was quenched by the addition of water (15 mL). Volatile carbon-14 radioactive components were flushed from the system with nitrogen gas and collected in a flask containing DMF, which was then frozen in a Dewar of liquid nitrogen and stoppered. The reaction mixture was filtered to remove molecular sieves and these were washed with ethyl acetate. The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was concentrated until a solid precipitated. Ethyl acetate (50 mL) was added. The solid was removed by vacuum filtration and washed with ethyl acetate (2 × 50 mL). The filtrates from both batches were combined and concentrated *in vacuo* to give a yellow oil. The residue was purified by flash chromatography eluting with 96:4 hexane:ethyl acetate. The combined batches gave 367 mCi of the title compound (75% yield). ^{1}H NMR ($CDCl_3$) δ6.93 (1 H, d, $J = 8.6$ Hz, H-6″), 6.63 (1H, d, $J = 12.3$ Hz, H-2), 6.49 (3H, m, H-2′, H-6′, H-5″), 6.43 (1H, d, $J = 12.2$ Hz, H-1), 4.70 (1H, sep, $J = 6.2$ Hz, C-2″ OC*H*(CH_3)$_2$), 4.43 (1H, sep, $J = 6.2$ Hz, C-3″ OC*H*(CH_3)$_2$), 3.82 (3H, s, C-4″ OCH_3), 3.79 (3H, s, C-4′ OCH_3), 3.65 (6H, s, C-3′, C-5′ OCH_3), 1.29 (12H, d, $J = 6.2$ Hz, C-2″, C-3″ OCH (CH_3)$_2$).

Synthesis of (*Z*)-1-[3′,[4′-^{14}C],5′-trimethoxyphenyl]-2-[2″,3″-dihydroxy-4″-methoxyphenyl]ethene; *Z*-[4′-^{14}C]CA1, (3)

(*Z*)-1-[3′,[4′-^{14}C],5′-Trimethoxyphenyl]-2-[2″,3″-di-[(*iso*propyl)oxy]-4″-methoxyphenyl] ethene (**2**) (1.35 g; 3.23 mmol; 183.7 mCi; 1.0 eq.) was dissolved in anhydrous dichloromethane (27 mL) and cooled to <0°C in an ice/acetone bath. $TiCl_4$ (1.57 mL; 14.19 mmol; 4.4 eq.) was added drop-wise to the reaction mixture, while maintaining temperature at <0°C, and stirred vigorously. The reaction mixture was stirred for 40 min at 0°C, and then quenched by slowly adding water, keeping the internal temperature <10°C. The aqueous layer was separated and extracted with dichloromethane. The combined organic layers were washed with brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4. This layer was concentrated on to silica and the compound was purified by flash chromatography through a silica column with a 0.5 cm plug of Florosil®, eluting with 75:25 hexane:ethyl acetate. The yellow oil was recrystallized from 50:50 hexane:ethyl acetate giving tan colored crystals. This reaction was performed in two batches of 183.7 mCi each and gave 100 mCi of the title compound (27% yield). ^{1}H NMR ($CDCl_3$) δ6.76 (1 H, d, $J = 8.7$ Hz, H-6″), 6.59 (1H, d, $J = 12.2$ Hz, H-2), 6.54 (3H, m, H-1, H-2′, H-6′), 6.38 (1H, d, $J = 8.7$ Hz, H-5″), 5.40 (2H, s, C-2″, C-3″ O*H*), 3.86 (3H, s, C-4′ OCH_3), 3.83 (3H, s, C-4′ OCH_3), 3.67 (6H, s, C-3′, C-5′ OCH_3).

Synthesis of (*Z*)-1-[3′,[4′-^{14}C],5′-trimethoxyphenyl]-2-[2″,3″-di-[([bis-[(benzyl)oxy]]phosphoryl)oxy]-4″-methoxyphenyl] ethene (4)

[4′-^{14}C]-CA1 (**3**) (0.58 g; 1.75 mmol; 100 mCi; 1.0 eq.) was dissolved in anhydrous acetonitrile (13 mL). The solution was cooled to −20°C and carbon tetrachloride (3.4 mL; 35 mmol; 20 eq.) was added. The reaction mixture was stirred for a few minutes, then di-*iso*propylethyl amine (1.2 mL; 7.0 mmol; 4.0 eq.) and 4-dimethylaminopyridine (42 mg; 0.34 mmol; 0.2 eq.) were added. After stirring for further 2 min, freshly prepared dibenzyl phosphite (1.16 mL; 5.22 mmol; 3.0 eq.) was added drop-wise and the reaction mixture stirred for 1 h. The reaction was quenched by the addition of 0.5 M KH_2PO_4 (4.2 mL) and ethyl acetate (16.9 mL). The organic phase was separated and washed with brine, then dried over Na_2SO_4. The organic layer was concentrated on the rotary evaporator to leave 1.8 g of yellow oil. This was purified by column chromatography on silica gel, eluting with 70:30 hexane:ethyl acetate. This gave 72.3 mCi of the title compound (72% yield). ^{1}H NMR ($CDCl_3$) δ7.24 (20 H, m, C-2″, C-3″ OP(O)($OCH_2C_6H_5$)$_2$), 7.00 (1H, d, J = 8.7 Hz, H-6″), 6.66 (1H, d, J = 8.5 Hz, H-5″), 6.65 (1H, d, J = 12.0 Hz, H-2), 6.51 (1H, d, J = 12.0 Hz, H-1), 6.45 (2H, s, H-2′, H-6′), 5.16 (4H, m, C-2″, C-3″OP(O)($OCH_2C_6H_5$)$_2$), 5.04–5.11 (4H, m, C-2″ C-3″ OP(O)-($OCH_2C_6H_5$)$_2$), 3.79 (3H, s, C-4″ OCH_3), 3.76 (3H, s, C-4′ OCH_3), 3.62 (6H, s, C-3′, C-5′ OCH_3).

Synthesis of (*Z*)-1-[3′,[4′-^{14}C],5′-trimethoxyphenyl]-2-[2″,3″-di-[(monopotassium)phosphate]-4″-methoxyphenyl] ethene; *Z*-[4′-^{14}C] -CA1P

The tetrabenzyl phosphate ester of [4′-^{14}C]CA1 (**4**) (0.52 g; 0.61 mmol; 35 mCi; 1.0 eq.) was dissolved in anhydrous acetonitrile (16.1 mL). The solution was cooled to −10°C and trimethylsilyl bromide (freshly distilled from CaH_2) (0.40 mL; 3.05 mmol; 5.0 eq.) was added drop-wise. The reaction mixture was stirred for 1.5 h at −10°C.

In a separate flask, potassium methoxide (0.43 g; 6.10 mmol; 10.0 eq.) was dissolved in anhydrous methanol (7.3 mL). The solution was cooled to −10°C and the phosphate solution was added slowly, drop-wise, over 2.5 h using a syringe pump. The reaction mixture was then allowed to warm to room temperature and stirred for 15 min. The solution was concentrated to dryness at 30°C on a rotary evaporator. The brown solid was dissolved in de-ionized water (3.2 mL) to give a cloudy, brown colored solution. The pH of the solution was measured at 13.4. The pH was carefully titrated to ~5.5 by drop-wise addition of 1.0 M aqueous HCl to the vigorously stirred solution. The titration was continued until pH 4.84 using 0.1 M HCl. The solution was then filtered to remove a brown scum and the flask and funnel were rinsed with de-ionized water (2.3 mL). Absolute ethanol (18 mL) was added to the filtrate causing a white solid to precipitate. The suspension was cooled at −20°C for 30 min, and then the solid was collected by vacuum filtration and washed with ethanol (6.5 mL). After 30 min air-drying in the funnel, a white solid, [4′-^{14}C]CA1P was obtained. This gave 20.5 mCi of the title compound (58% yield). ^{1}H NMR (D_2O) δ6.84 (1H, d, J = 8.7 Hz, H-6″), 6.68 (1H, d, J = 11.9 Hz, H-2), 6.64 (1H, d, J = 8.8 Hz, H-5″) 6.61 (2H, s, H-2′, H-6′), 6.58 (1H, d, J = 12.0 Hz, H-1), 3.76 (3H, s, C-4″ OCH_3), 3.68 (3H, s, C-4′ OCH_3), 3.62 (6H, s, C-3′, C-5′ OCH_3); HPLC retention time is 16.82 min (consistent with an authentic sample); 97.5% chemical purity by HPLC area % (UV), 96.7% radiochemical purity by HPLC area %, specific activity 55 mCi/mmol (determined by gravimetry).

Acknowledgement

Almac Sciences gratefully acknowledge sponsorship of this work by OXiGENE and also thank Professor Kevin Pinney and Dr Madhavi Sriram of Baylor University for helpful discussions throughout the course of this project and supply of (*Z*)-1-[3′,5′-dimethoxy-4′-[(*tert*-butyldimethylsilyl)oxy]phenyl]-2-[2″,3″-di-[(*iso*-propyl)oxy]-4″-methoxyphenyl] ethene (**1**). K. G. P. is appreciative of the support from OXiGENE Inc. and The Welch Foundation (Grant No. AA-1278) that funded, in part, the original synthetic methodology development (with carbon-12).

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