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Synthesis of dialkylaminoaryl phosphonate bioisosteres of tyrosine and tyramine: a novel application of allene phosphonate chemistry for the synthesis of false substrates of tyrosinase

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ABSTRACT

Tyrosinase is the rate-limiting oxidase in the synthesis of melanin, making it an obvious target for the treatment of melanotic melanomas. Tyrosine and tyramine are its natural substrates, but many of their derivatives are inhibitors or false substrates, and are therefore prime candidates for melanoma chemotherapy. A series of dialkylphosphonate derivatives of tyramine have now been synthesized in order to extend the chemical diversity of tyrosinase substrates. The known reactivity between alkenephosphonates and nucleophiles was exploited by the addition of 4-(2-aminoethyl)phenol (tyramine) across the 2,3-double bond of 1,2-alkadiene phosphonates, to obtain the desired bisphosphonate derivatives. These reactions were highly chemoselective and regioselective but not stereoselective. Five of the reported novel dialkylphosphonate aminophenols were substrates for mushroom tyrosinase *in vitro*: dimethyl 2-[2(4-hydroxyphenyl)ethylamino]-3-methyl-1-butenephosphonate (**3**);diethyl 2-[2(4-hydroxyphenyl)ethylamino]-3-methyl-1-butenephosphonate (**4**);dimethyl 2-[2-(4-hydroxyphenyl)ethylamino]-2-cyclohexyl-1-ethenephosphonate (**5**);diethyl 2-[2-(4-hydroxyphenyl)ethylamino]-2-cyclohexyl-1-ethenephosphonate (**6**);diethyl 2-[2-(4-hydroxyphenyl)ethylamino]ethanephosphonate (**7**). Compound **3** blocked the pigmentation of anagen hair *in vivo* in a murine animal model, a further demonstration that these compounds are able to enter and disrupt the melanogenic pathway.

INTRODUCTION

Malignant melanoma is among the most aggressive and drug resistant cancers. It arises via a poorly understood interplay of host-environment interactions and risk factors. Sunlight, mainly UV B (280-320 nm), appears to be one of the most important environmental causative factors, although the relationship between sun exposure and the development of melanoma is not simple (Etzkorn *et al*., 2013; Braeuer *et al*., 2014). Localized melanoma can be managed and most cases even cured by early surgical ablation (Urist, 1996) but treatment for the disseminated disease remains elusive. An extensive literature describes clinical and experimental approaches to the chemotherapy, immunotherapy, endocrine therapy and molecular approaches to control melanoma (Garbe *et al*., 2011; An *et al*., 2009). Chemotherapy based on alkylating agents (e.g., dacarbazine; DTIC), nitrosoureas and platinum complexes all have low response rates, are moderately to severely toxic, and have serious side-effects. In short, there is no accepted standard regimen for the treatment of metastatic melanoma (Voskoboynik and Arkenau, 2014; NCI, 2013). One of the defining characteristics of most melanomas is an increase in melanin pigmentation, making melanin an obvious target for anti-melanoma therapy (Slomonski *et al*., 2015; Farmer *et al*., 2003). The production and distribution of melanin (melanogenesis) by melanocytes in skin and hair follicles involves packaging melanin in specialized lysosome-like organelles (melanosomes) which contain several enzymes that mediate the production of melanin. Tyrosinase (TYR) initiates melanin synthesis by catalyzing the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to

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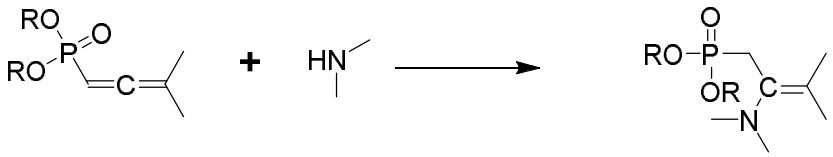
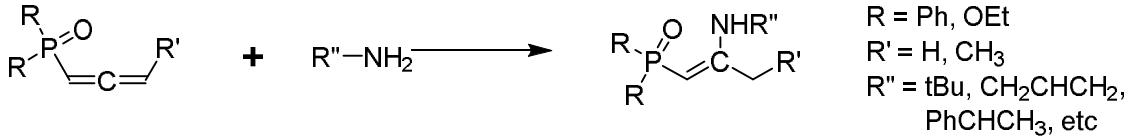


Scheme 1. Reaction of amines at the 1,2-double bond of dialkyl butadienephosphonates to form the respective 1,2-adducts. (Pudovik andKhusainova, 1966)



Scheme 2. Nucleophilic addition of amines at the 2,3 double bond of dialkyl butadienephosphonates to form the respective 2,3-adducts (Palacios *et al*, 1996).

to DOPA quinone. The pathway diverges to either eumelanin (red and amber) or pheomelanin (black), depending on whether dopaquinone interacts with cysteine or other related sulfhydryl-containing moieties. Because the melanogenic pathway is found only in melanocytes, it is an attractive, selective target not only for therapeutic intervention in cancer but also for cosmetic control of skin pigmentation (Speeckaert *et al*., 2014; Regad, 2013). Tyrosinase inhibitors or false substrates of tyrosinase represent one approach to the development of anti-melanin drugs (Jimbow *et al*., 1993). Several classes of tyrosinase inhibitors / false substrates with potential antimelanotic activity have been identified, including 2- and 4-sulfur-substituted phenols (Riley *et al*, 1997; Jimbow *et al*., 2011).

The literature is replete with reports of the use of bisphosphonate drugs for treating osteoporosis (Reid, 2011) and to a lesser extent, of nucleotide phosphonate analogues for the treatment of viral disease (Yim and Hwang, 2013). However, there are very few other reported applications of phosphonates, or indeed phosphorous-containing drugs in modern medicine. As early as the 1960's, it was reported that acetylene phosphines obtained through the reaction of phosphorus trichloride with α-acetylenic alcohols spontaneously isomerized under mild conditions to form allenephosphonates (Ignat'ev *et al*., 1969; Angelov and Neilson, 2992; Neilson and Angelov, 2009). Given that phosphorylated allenes are quite reactive and interact with both electrophilic and nucleophilic reagents, this simple method provided a synthetic route to many compounds with a range of substituents on phosphorus. The reactions with electrophiles have been studied in detail (Khusainova and Pudovik, 1987; Angelov, 1983; Khusainova and Pudovik, 1978; Alabigin and Brel, 1997), while interactions with nucleophiles have been less thoroughly explored. One of the historical reasons for the limited nucleophilic chemistry with these compounds is that most nucleophilic reagents require the use of a catalyst to drive the reaction. An exception to this rule is found in the reactions with amines, where allene phosphorus compounds react directly. The first example of the reaction of amines with substituted allenephosphonic acid derivatives was published in 1966 (Pudovik and Khusainova, 1966) when it was shown that diethylamine and piperidine add to dialkyl 3-methyl-1,2-butadienephosphonates on the α-double bond to afford 1,2-aducts (Scheme 1).

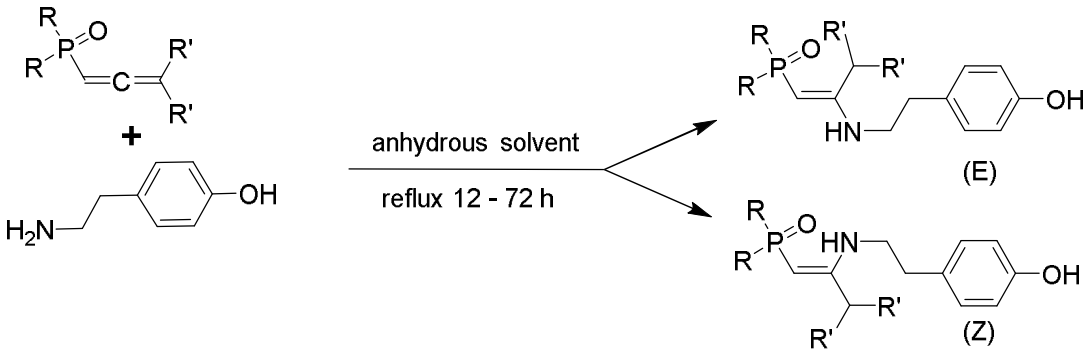
More recently, nucleophilic additions to allene phosphonium salts, phosphine oxides and diethyl allene phosphonate have been reported to yield a wide variety of primary amines. For example, addition of aminoethanol occurs at the β-double bond, with isolation of the 2,3-adducts in all cases (Scheme 2) (Palacios *et al*, 1996). The current work is based on the premise that incorporation of phosphonate moieties into the alkylamino portion of the tyrosinase substrates could potentially open the door to novel antimelanotic compounds through changes in target enzyme selectivity, pharmacokinetics, target-selective delivery and processing along the melanogenic pathway. The synthesis of the novel phosphonate derivatives now reported is based on the reactivity between amines, specifically tyramine analogues, and dialkylallene phosphonates. The design and synthesis of several structural phosphonate analogues of tyrosine is now reported, along with preliminary evidence for their oxidation by tyrosinase and their inhibition of melanogenesis.

RESULTS AND DISCUSION

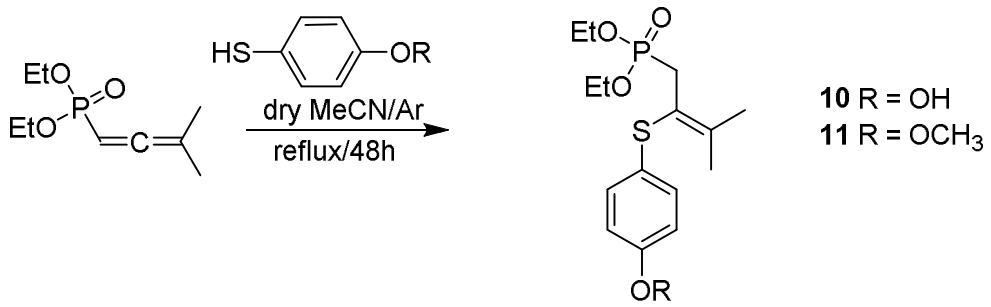
Chemistry

The objective of the current investigations was to synthesize N-substituted tyramine phosphonates, which in theory could proceed via either or both the amino and hydroxyl groups. The high acidity of the phenolic hydroxyl could potentially promote reaction with the allenic system of 1,2-alkadiene phosphonates via an electrophilic mechanism (Alabugin and Brel, 1997), but current experimental data from reactions between several 1,2-alkadiene phosphonates and 4-(2-aminoethyl)phenol (tyramine) show that the reaction is highly chemo- and regioselective, but not stereoselective, and proceeds only by addition of the amino group to the 2,3-double bond (Scheme 3).

Experimentally, 1,2-alkadiene phosphonates were heated with amines under reflux in acetonitrile (Table 1). Reaction progress was monitored with ^{31}P-NMR. After removing the solvent, crude product could be recrystallized as a solid compound.



Scheme 3. General reaction of amines with dialkyl 1,2-alkadienephosphonates to prepare dialkyl aminophosphonates. In this scheme, tyramine attack at C2 of the alkadiene system affords the *E/Z* isomers by introduction of the amino substituent only at the central alkadiene carbon. The reactions are carried out under reflux in anhydrous solvent. Reaction products are worked up by removal of the solvent under reduced pressure, and the products are isolated and purified by recrystallization or distillation under vacuum.



Scheme 4. Reaction of aromatic thiols with diethyl 3-methyl-1,2-butadienephosphonate to form compounds **10** and **11**.

In all cases, the 2,3-adduct was obtained as a mixture of *E* and *Z* isomers (Scheme3), but it was not possible to separate either isomer in pure form by recrystallization. Thus, in a representative case, the ^{31}P-NMR spectrum of crude reaction product for **4**, for example, included two peaks at δ_P 29.07 (*E*-isomer) and 29.6 (*Z*-isomer) ppm (Palacios *et al*., 1996), in an approximate 2:1 isomeric ratio. After repeated recrystallization, the ratio was approximately 6:1 in favor of the *E*-isomer. The ^{1}H and ^{13}C NMR spectra also displayed two groups of signals, confirming the structures as *E*- and Z-isomer mixtures.

The reaction of 1,2-alkadiene phosphonates with thiols was also investigated briefly. Reaction of diethyl 3-methyl-1,2-butadienephosphonate with either 4-methoxythiophenol or 4-hydroxythiophenol afforded diethyl 2(4-hydroxyphenylthio)-3-methyl-2-butenephosphonate (**10**) and diethyl 2-(4-methoxyphenylthio)-3-methyl-2-butenephosphonate (**11**), respectively (Scheme 4). Both gave rise to 1,2-adducts only, and in each case, a mixture of *E* and *Z* isomers was obtained.

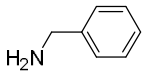
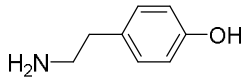
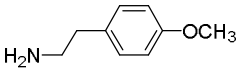
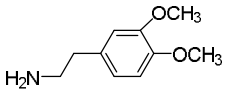
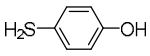
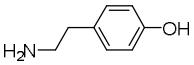
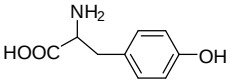
In vitro cytotoxicity

MTT cell survival assays were implemented using test compounds at concentrations from $1x10^{-1}$ to $1x10^{-7}$ mM. There was no evidence of growth inhibition at concentrations below 0.1 mM (data not shown). The low cost and convenience of the MTT assay is offset by caveats including its tendency to overestimate survival, its relative insensitivity to small changes and, most importantly, it measures mitochondrial activity (i.e. metabolism) rather than viability *per se*. Although the MTT assay is a convenient screening procedure, interested readers are referred to Cobb's review on cell viability testing (Cobb, 2013).

Oxidation by mushroom tyrosinase

A number of antimicrobial and anticancer α-aminophosphonates (Dake *et al*., 2011; Gu and Jin, 2012; Abdel-Megeed *et al*., 2012) and β-keto- and β-hydroxy-phosphonates (Cui *et al*., 2008) have been reported, but none have been tested against tyrosinase. Nine of the currently-reported phosphorylated amines were incubated with tyrosinase, and of these, five (**3-7**) were mushroom tyrosinase substrates *in vitro* as judged by visual inspection and UV-vis spectrophotometry. In this screen, active compounds developed a light brownish color (Plate 1A) and display an additional UV absorbance peak at 289-292 nm (data not shown). As anticipated, amines which contained a blocked aromatic hydroxyl group (**8**, **9**), or no phenolic hydroxyl (**1**, **2**), were inactive in these tests. This finding demonstrates the vital nature of the aromatic hydroxyl group in the design of tyrosinase substrates and false substrates and shows that the phosphonate moiety does not interfere with tyrosinase-mediated oxidation of the phenolic component of these molecules. The natural tyrosinase substrates (tyramine; tyrosine) served as positive controls in these experiments, and solvent and unreacted diene phosphonates provided negative results, thereby confirming that the observed effects were due to test compound-enzyme interaction (Table 1).

Table 1: Structural elements of tyrosinase-directed aminoaryldialkylphosphonate bioisosteres of tyrosine.

S. No.	Compound	Precursor Amine	Precursor Phosphonate	Tyrosinase assay
1	Dimethyl 2-benzylamino-3-methyl-1-butene phosphonate	 benzylamine	dimethyl 3-methyl-1,2-butadiene phosphonate	colorless
2	Diethyl 2-benzylamino-3-methyl-1-butene phosphonate		diethyl 3-methyl-1,2-butadiene phosphonate	colorless
3	Dimethyl 2-[2(4-hydroxy-phenyl)-ethylamino]-3-methyl-1-butene phosphonate		dimethyl 3-methyl-1,2-butadiene phosphonate	light brown
4	Diethyl 2-[2(4-hydroxy-phenyl)-ethylamino]-3-methyl-1-butenephosphonate	 4-hydroxyphenethylamine (tyramine)	diethyl 3-methyl-1,2-butadiene phosphonate	light brown
5	Dimethyl 2-[2-(4-hydroxy-phenyl)ethylamino]-2-cyclohexyl-1-ethene phosphonate		dimethyl 2-cyclo-hexylidene ethane phosphonate	light brown
6	Diethyl 2-[2-(4-hydroxy-phenyl)-ethylamino]-2-cyclohexyl-1-ethene phosphonate		diethyl 2-cyclohexylidene-ethane phosphonate	light brown
7	Diethyl 2-[2-(4-hydroxy-phenyl)-ethylamino]-ethane phosphonate		diethyl vinyl phosphonate	light brown
8	Diethyl 2-[2-(4-methoxy-phenyl)ethylamino]-3-methyl-1-butene phosphonate	 4-OMe-phenethylamine	diethyl 3-methyl-1,2-butadiene phosphonate	colorless
9	Diethyl 2-[2-(3,4-dimethoxy-phenyl)ethyl-amino]-3-methyl-1-butene phosphonate	 dimethoxyphenethylamine	diethyl 3-methyl-1,2-butadiene phosphonate	colorless
10	Diethyl 2-(4-hydroxy-phenylthio)-3-methyl-2-butene phosphonate		diethyl 3-methyl-1,2-butadiene phosphonate	not tested
11	Diethyl 2(4-methoxy-phenylthio)-3-methyl-2-butene phosphonate	4-hydroxybenzenethiol (4-hydroxythiophenol)	diethyl 3-methyl-1,2-butadiene phosphonate	not tested
12	Bis(methylazeridinyl) 3-methyl-1,2-butadiene phosphonate	phosphonate controls		colorless
13	Dimethyl 2-cyclohexylidene ethane phosphonate			colorless
14	PBS	solvent control	negative control	colorless
15	Tyramine*	 4-hydroxyphenethylamine (tyramine)	positive control	light brown
16	Tyrosine*	 4-hydroxyphenylalanine (tyrosine)	positive control	light brown

*natural tyrosinase substrates

Inhibition of *in vivo* melanin biosynthesis

A preliminary study of compound **3** in a C57BL/6J mouse provided a clear indication that these compounds are false substrates of tyrosinase once they enter the melanogenic pathway. Black hair were manually plucked from a small region on the backs of mice in order to stimulate new follicular melanocytes and increase tyrosinase activity. This allows visualization of the blockage of melanin synthesis through the regrowth of unpigmented (white) new (anagen) hair (Plate 1B). In this test, the mouse was given daily intraperitoneal (i.p.) injections of **3** (0.878 mmol/ 300 mg/kg) daily for 14 days, a dosage regimen based on literature data for other putative false substrates (Ito *et al*., 1987).

None of the amino- or thiol adducts were toxic to either G361 or SK-MEL-24 melanoma cells in cell culture at inhibitory concentrations (IC_{50}) below $0.5x10^{-4}$ M. This low toxicity was also evident in the *in vivo* study, although the *in vivo* experiment was not intended to provide toxicity data.

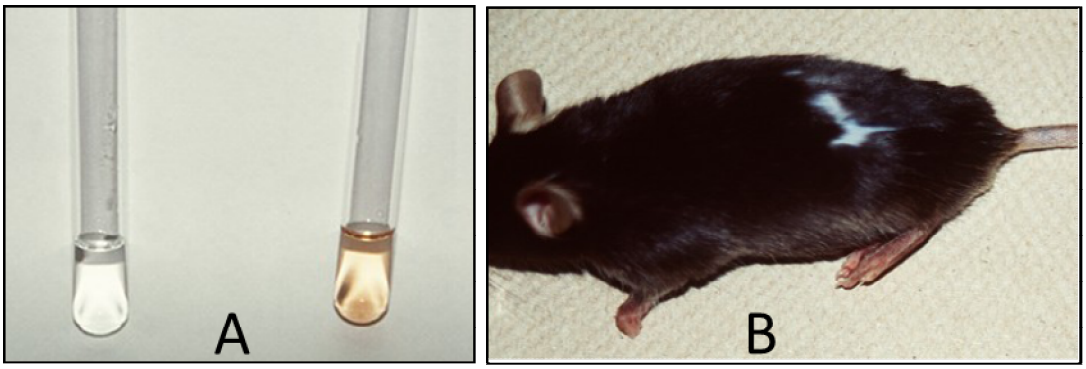


Plate 1. A: Color generation following oxidation of a tyrosinase substrate (left) and non-substrate (right) in the presence of mushroom tyrosinase. B: Inhibition of melanogenesis in new (anagen) hair growing on the back of a C57BL/6J mouse dosed with **3** (i.p. dose 300 mg/kg/d x 14 d).

In conclusion, the facile synthesis of dialkyl aminoarylphosphonates as potential substrates of tyrosinase proceeded by reaction of the appropriate amine (i.e., tyramine, 4-methoxyphenethyl amine orbenzylamine) with dialkyl 1,2-alkadienephosphonates. In contrast, the alkyl amines afforded only 2,3-adducts and the thiophenols produced only 1,2-adducts under similar reaction conditions. Five of the amino adduct tyramine derivatives, **3, 4, 6, 7** and **8** tested positive for oxidation by mushroom tyrosinase, and compound **3**, in a preliminary study inhibited melanogenesis *in vivo* in a murine model.

EXPERIMENTAL

Materials and methods

All chemical manipulations were performed under an atmosphere of dry argon. The 1,2-alkadienephosphonate precursors were prepared by a known procedure (Ignat'ev *et al*., 1969); all other starting compounds were obtained from commercial sources (Aldrich) and were distilled or recrystallized before use. Solvents were dried and distilled before use and stored over molecular sieves. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AM 300 instrument operating at 300.13, 75.47 and 121.53 MHz for the respective nuclei using 85 % H_3PO_4 and $Si(CH_3)_4$ as external standards.

In all spectroscopic studies $CDCl_3$ was used as both a solvent and as an internal lock. Positive shifts lie downfield of the standard in all cases.

Chemical syntheses

General Procedure

A suitable round bottom flask equipped with a stir bar, rubber septum and argon inlet was charged with distilled anhydrous solvent (stored over molecular sieves) and the respective dialkyl phosphonate and amineprecursors.

The reaction mixture was heated under reflux, usually overnight, until ^{31}P NMR showed the reaction was complete. The solvent was removed under reduced pressure and the residue was worked up by direct recrystallization, chromatography followed by recrystallization, or distillation under vacuum followed by crystallization to obtain pure product.

Dimethyl 2-benzylamino-3-methyl-1-butenephosphonate (**1**)

A solution of anhydrous distilled acetonitrile (7 mL), dimethyl 3-methyl-1,2-butadienephosphonate (5 mmol, 0.88 g) and distilled benzylamine was heated under reflux for 3 h. The solvent was removed under reduced pressure to yield a solid residue which was fractionally distilled twice under vacuum. *^{1}H-NMR:* 1.10 d and 1.17 d (6H, $^{3}J_{HH}$ 6.7 Hz, $^{3}J_{HH}$ 7.0 Hz, *E*- and *Z*-CH_3); 2.61 – 2.82 m (1H, *E*- and *Z*-CH); 3.64 d and 3.67 d (3H, $^{3}J_{PH}$ 11.0 Hz, *E*- and *Z*-CH); 3.73 d and 3.77 d (1H, $^{2}J_{PH}$ 11.3 Hz, $^{2}J_{PH}$ 9.4 Hz, *E*- and *Z*-=CH); 4.17 d and 4.37 d (2H, $^{3}J_{HH}$ 5.2 Hz, $^{3}J_{HH}$ 6.4 Hz, *E*- and *Z*-CH_2); 4.45 t (*E*-NH) and 7.75 t (*Z*-NH); 7.25 – 7.39 mm (arom); *^{13}C-NMR:* 21.5 s and 21.85 s (2C, *E*- and *Z*-CH_3-CH); 29.38 d and 30.26 d (1C, $^{3}J_{PC}$ 17.5 Hz, $^{3}J_{PC}$ 4.4 Hz, *E*- and *Z*-CH-CH_3); 46.12 s and 47.23 s (1C, *E*- and *Z*-CH_2-arom); 51.56 d and 51.60 d (2C, $^{2}J_{PC}$ 3.4 Hz, $^{2}J_{PC}$ 2.4 Hz, *E*- and *Z*-CH_3-O); 65.13 d and 71.11 d (1C, $^{1}J_{PC}$ 195.6 Hz, $^{1}J_{PC}$ 216.4 Hz, *E*- and *Z*-=CH-P); 126.26 – 139.46 (C-arom); 167.88 d and 174.1 d (1C, $^{2}J_{PC}$ 20.8 Hz, $^{2}J_{PC}$ 4.6 Hz, *E*- and *Z*-=C-NH); *^{31}P-NMR:* 29.14 (*E*-isomer) and 21.78 (*Z*-isomer); Anal. calc. for $C_{14}H_{22}NO_3P$: C-59.25 %, H-7.85%, N-4.95%; found: C-59.21%, H-7.83%, N-4.93%.

Diethyl 2-benzylamino-3-methyl-1-butenephosphonate (**2**)

A solution of anhydrous distilled ethanol (7 mL), diethyl 3-methyl-1,2-butadienephosphonate (5 mmol, 1.0 g) and distilled benzylamine was heated under reflux overnight. The solvent was removed under reduced pressure to yield a yellow solid which was fractionally distilled twice under vacuum. Yield 75%; bp 140-150^{o}C/ 0.05 mm Hg; *^{1}H-NMR:* 1.03 d and 1.12 d (6H, $^{3}J_{HH}$ 7.0 Hz, $^{3}J_{HH}$ 6.7 Hz, *E*- and *Z*-CH_3); 1.21 t and 1.26 t (6H, $^{3}J_{HH}$ 7.0 Hz, $^{3}J_{HH}$ 6.7 Hz, *E*- and *Z*-CH_3); 2.45 – 2.78 m (1H, *E*- and *Z*-CH); 3.36 d and 3.72 d (1H, $^{2}J_{PH}$ 11.3 Hz, $^{2}J_{PH}$ 10.0 Hz, *E*- and *Z*-CH); 3.86 – 4.02 m (4H, *E*- and *Z*-CH); 4.12 d and 4.30 d (2H, $^{3}J_{HH}$ 5.2 Hz, $^{3}J_{HH}$ 6.7 Hz, *E*- and *Z*-CH_2); 4.75 t (*E*-NH,) and 7.74 t (*Z*-NH); 7.19 – 7.28 mm (arom); *^{13}C-NMR:* 16.13 s and 16.21 s (2C, *E*- and *Z*-CH_3-CH); 20.86 s and 21.72 s (2C, *E*- and *Z*-CH_3); 29.2 d and 30.0 d (1C, $^{3}J_{PC}$ 17.8 Hz, $^{3}J_{PC}$ 4.6 Hz, *E*- and *Z*-CH); 46.09 s and 47.01 s (1C, *E*- and *Z*-CH_2-arom); 60.45 d and 60.58 d (2C, $^{2}J_{PC}$ 5.5 Hz, $^{2}J_{PC}$ 5.4 Hz, *E*- and *Z*-CH_2-O); 67.62 d and 72.85 d (1C, $^{1}J_{PC}$ 193.4 Hz, $^{1}J_{PC}$ 215.4 Hz, *E*- and *Z*-=CH-P); 126.09 – 139.55

(C-arom); 167.14 d and 172.9 d (1C, $^{2}J_{PC}$ 17.5 Hz, $^{2}J_{PC}$ 4.4 Hz, *E*- and *Z*-=C-NH); *^{31}P-NMR:* 28.61 (*E*-isomer) and 28.98 (*Z*-isomer); Anal. calc. for $C_{16}H_{26}NO_3P$: C-61.72 %, H-8.42%, N-4.50%; found: C-61.33%, H-8.50%, N-4.47%.

Dimethyl 2-[2(4-hydroxyphenyl)ethylamino]-3-methyl-1-butenephosphonate (**3**)

A solution of anhydrous acetonitrile (10 mL), dimethyl 3-methyl-1,2-butadienephosphonate (6 mmol, 1.05 g) and tyramine (16 mmol, 0.82 g) was heated under reflux overnight, the solvent was removed to yield a viscous semi solid residue which was dissolved in dichlormethane:methanol (3 mL; 2%) and separated on a neutral alumina column (15 g, 60-325 mesh, Fisher) with dichlormethane:methanol (2%). The main fraction was dried and the residue was recrystallized from dichlormethane:methanol and hexane to obtain a lightly yellow solid. Yield 77%; mp 145-147^{o}C;*^{1}H-NMR:* 0.98 d and 1.06 d (6H, $^{3}J_{HH}$ 7.0 Hz, $^{3}J_{HH}$ 6.7 Hz, *E*- and *Z*-CH_3-CH); 2.54 m (1H, *E*- and *Z*-CH-CH_3); 2.67 t and 2.76 t (2H, $^{3}J_{HH}$ 7.0 Hz, *E*- and *Z*-CH_2-arom); 3.16 dt and 3.29 dt (2H, $^{3}J_{HH}$ 7.0 Hz, $^{3}J_{HH}$ 12.2 Hz (NH-CH_2), *E*- and *Z*-CH_2-NH); 3.62 d and 3.78d (6H, $^{3}J_{PH}$ 11.3 Hz, *E*- and *Z*-CH_3-O); 3.73 s and 3.79 d (1H, $^{2}J_{PH}$ 11.3 Hz, *E*- and *Z*-=CH); 4.29 dt (1H, $^{3}J_{HH}$ 11.9 Hz, $^{4}J_{PH}$ 6.8 Hz, *E*-NH) and 7.10 t (1H, $^{3}J_{HH}$ 6.1 Hz, *Z*-NH); 6.80 – 7.0 m (4H, arom); 8.4 bs (1H, *E*- and *Z*-OH); *^{13}C-NMR:* 21.00 s and 21.73 s (2C, *E*- and *Z*-CH_3-CH); 29.67 d and 30.64 d (1C, $^{3}J_{PC}$ 17.6 Hz, $^{3}J_{PC}$ 4.4 Hz, *E*- and *Z*-CH-CH_3); 33.18 s and 36.32 s (1C, *E*- and *Z*-CH_2-arom); 43.98 s and 44.46 s (1C, *E*- and *Z*-CH_2-NH); 51.77 d and 51.92 d (2C, $^{2}J_{PC}$ 5.6 Hz, $^{2}J_{PC}$ 5.5 Hz, *E*- and *Z*-CH_3-O); 62.744 d and 68.67 d (1C, $^{1}J_{PC}$ 196.7 Hz, $^{1}J_{PC}$ 218.6 Hz, *E*- and *Z*-=CH-P); 115.28 – 129.60, and 174.10 (C-arom); 155.91 d and 168.59 d (1C, $^{2}J_{PC}$ 24.2 Hz, $^{2}J_{PC}$ 20.9 Hz, *E*- and *Z*-=C-NH); *^{31}P-NMR:* 31.13 and 32.50 (*E*- and *Z*-isomer); Anal. calc. for $C_{17}H_{28}NO_4P$: C-57.50%, H-7.72%, N-4.47%; found C-53.15, 52.93%, H-7.48, 8.07%, N-4.24, 4.20%.

Diethyl 2-[2(4-hydroxyphenyl)ethylamino]-3-methyl-1-butenephosphonate (**4**)

A solution of anhydrous acetonitrile (3 mL), diethyl 3-methyl-1,2-butadienephosphonate (3 mmol, 0.62 g) and tyramine (3 mmol, 0.41 g) was heated under reflux overnight, the solvent was removed under reduced pressure, and the tan colored residue was recrystallized from hexane/ CH_2Cl_2. Yield 78%; mp 97-99^{o}C; *^{1}H-NMR:* 0.89 d and 0.98 d (6H, $^{3}J_{HH}$ 6.7 Hz, *E*- and *Z*-CH_3-CH); 1.19 t and 1.21 t (6H, $^{3}J_{HH}$ 7.0 Hz, *E*- and *Z*-CH_3-CH_2); 2.47 m (1H, *E*- and *Z*-CH-CH_3); 2.56 t and 2.67 t (2H, $^{3}J_{HH}$ 7.1 Hz, *E*- and *Z*-CH_2-arom); 3.08 dt and 3.18 dt (2H, $^{3}J_{HH}$ 7.1 Hz, $^{3}J_{HH}$ 11.9 Hz (NH-CH_2), *E*- and *Z*-CH_2-NH); 3.41 d and 3.68 d (1H, $^{2}J_{PH}$ 11.1 Hz, *E*- and *Z*-CH); 3.90 m (4H, *E*- and *Z*- CH_2-O); 4.03 t (1H, $^{3}J_{HH}$ 11.9 Hz, *E*-NH) and 7.09 t (1H, $^{3}J_{HH}$ 6.1 Hz, *Z*-NH); 6.79 m (arom); *^{13}C-NMR:* 16.29 s and 16.37 s (2C, *E*- and *Z*-CH_3-CH_2); 20.2 s and 20.98 s (2C, *E*- and *Z*-CH_3-CH); 29.58 d and 30.51 d (1C, $^{3}J_{PC}$ 17.3 Hz, $^{3}J_{PC}$ 4.3 Hz, *E*- and *Z*-CH-CH_3); 33.29 s and 36.41 s (1C, *E*- and *Z*-CH_2-arom); 44.00 s and 44.46 s (1C, *E*- and *Z*-CH_2-NH); 61.06 d and 62.36 d (2C, $^{2}J_{PC}$ 5.5 Hz, $^{2}J_{PC}$ 6.6 Hz, *E*- and *Z*-CH_2-O); 65.04 d and 70.79 d (1C, $^{1}J_{PC}$ 196.6 Hz, $^{1}J_{PC}$ 218.7 Hz, *E*- and *Z*-=CH-P); 115.32 – 115.83, 128.82 – 129.79, and 173.18 (C-arom); 155.85 d and 167.88 d (1C, $^{2}J_{PC}$ 26.4 Hz, $^{2}J_{PC}$ 20.9 Hz, *E*- and *Z*-=C-NH); *^{31}P-NMR:* 29.07 and 29.60 (*E*- and *Z*-isomer); Anal. calc. for $C_{17}H_{28}NO_4P$: C-59.81%, H-8.27%, N-4.10%; found: C-59.49%, H-8.42%, N-4.11%.

Dimethyl 2-[2-(4-hydroxyphenyl)ethylamino]-2-cyclohexyl-1-ethenephosphonate (**5**)

A solution of anhydrous acetonitrile (10 mL), dimethyl 2-cyclohexylideneethenephosphonate (10 mmol, 2.16 g) and tyramine (10 mmol, 1.37 g) was heated under reflux for two days. Upon cooling, a tan colored precipitate formed. The reaction mixture was reheated until the precipitate dissolved, treated with activated carbon and then gravity filtered through Whatman paper. The resulting solid was recrystallized from acetonitrile. Yield 59%; mp 139-141^{o}C; *^{1}H-NMR:* 1.1 – 1.8 m (11H, *E*- and *Z*-C_6H_{11}); 2.69 t and 2.78 t (2H, $^{3}J_{HH}$ 6.1 Hz, *E*- and *Z*-CH_2-arom); 3.13 dt and 3.30 dt (2H, $^{3}J_{HH}$ 7.5 Hz, *E*- and *Z*-CH_2-NH); 3.46 d and 3.80 d (1H, $^{2}J_{PH}$ 11.6 Hz, *E*- and *Z*-CH); 3.63 d and 3.69 d (6H, $^{3}J_{PH}$ 11.3 Hz *E*- and *Z*- CH_3-O); 4.22 t (1H, $^{3}J_{HH}$ 5.8 Hz, *E*-NH) and 7.18 t (1H, $^{3}J_{HH}$ 5.7 Hz, *Z*-NH); 6.79 – 7.02 m (4H, arom); 8.08 bs (1H, OH-arom), *^{13}C-NMR:* 23.54 – 26.55, 32.43, 33.21, 36.47, 41.32 (6C, *E*- and *Z*-C_6H_{11}); 31.73 s and 31.66 s (1C, *E*- and *Z*-CH_2-arom); 44.21 s and 44.43 s (1C, *E*- and *Z*-CH_2-NH); 51.79 s and 51.91 d (2C, $^{2}J_{PC}$ 5.5 Hz, *E*- and *Z*-CH_3-O); 64.0 d and 70.3 d (1C, $^{1}J_{PC}$ 198.1 Hz, $^{1}J_{PC}$ 215.6 Hz, *E*- and *Z*-=CH-P); 115.66 and 129.39 – 129.70 (C-arom); 155.5 d and 167.6 d (1C, $^{2}J_{PC}$ 21.2 Hz, $^{2}J_{PC}$ 21.2 Hz, *E*- and *Z*-=C-NH); *^{31}P-NMR:* 33.47 and 33.91 (*E*- and *Z*-isomer); Anal.calc. for $C_{18}H_{28}NO_4P$: C-61.17%, H-7.99%, N-3.94%; found C-60.90%, H-8.05%, N-4.10%.

Diethyl 2-[2-(4-hydroxyphenyl)ethylamino]-2-cyclohexyl-1-ethenephosphonate (**6**)

A solution of anhydrous acetonitrile (15 mL), diethyl 2-cyclohexylideneethenephosphonate (15 mmol, 3.7 g) and tyramine (15 mmol, 2.1 g) was heated under reflux for three days. The solvent was removed under reduced pressure to yield a tan colored solid, which was recrystallizedfrom acetonitrile. Data for **3**: yield 42%; mp 127-129^{o}C;*^{1}H-NMR:* 1.06 m and 1.72 m (C_6H_{11}); 1.28 t and 1.30 t (6H, $^{3}J_{HH}$ 7.1 Hz, *E*- and *Z*-CH_3-CH_2); 2.65 t and 2.74 t (2H, $^{3}J_{HH}$ 7.1 Hz, *E*- and *Z*-CH_2-arom); 3.13 dt and 3.24 dt (2H, $^{3}J_{HH}$ 7.2 Hz, $^{3}J_{HH}$ 12.2 Hz (NH-CH_2), *E*- and *Z*-CH_2-NH); 3.49 d and 3.75 d (1H, $^{2}J_{PH}$ 11.0 Hz, *E*- and *Z*-CH); 4.04 m (4H, *E*- and *Z*-CH_2-O); 4.22 t (1H, $^{3}J_{HH}$ 5.2 Hz, *E*-NH) and 7.14 t (1H, $^{3}J_{HH}$ 5.1 Hz, *Z*-NH); 6.81 – 6.98 m (4H, arom); 8.62 bs (1H, OH-arom), *^{13}C-NMR:* 16.24 d and 16.33 d (2C, $^{3}J_{PC}$ 3.3 Hz, $^{3}J_{PC}$ 3.3 Hz, *E*- and *Z*-CH_3-CH_2); 26.02 – 26.41, 32.26, 33.21, 40.25, 41.18 (6C, *E*- and *Z*-C_6H_{11}); 31.49 s and 36.40 s (1C, *E*- and *Z*-CH_2-arom); 44.10 s and 44.45 s (1C, *E*- and *Z*-CH_2-NH); 61.02 d and 62.36 s (2C, $^{2}J_{PC}$ 5.5 Hz, *E*- and *Z*-CH_2-O); 64.2 d and 70.6 d (1C, $^{1}J_{PC}$ 197.2 Hz, $^{1}J_{PC}$ 217.9 Hz, *E*- and *Z*-=CH-P); 115.62, 128.66 – 129.67, and 172.12 (C-arom); 155.8 d and 167.3 d (1C, $^{2}J_{PC}$ 22.0 Hz, $^{2}J_{PC}$ 21.9

Hz, *E*- and *Z*-=C-NH); *[31]P-NMR:* 27.64 and 29.71 (*E*- and *Z*-isomer); Anal.calc. for $C_{20}H_{32}NO_4P$: C-62.97%, H-8.46%, N-3.67%; found C-62.89%, H-8.89%, N-3.85%.

Diethyl 2-[2-(4-hydroxyphenyl)ethylamino]-ethanephosphonate (**7**)

A suspension of potassium carbonate (7.2 mmol, 1 g), anhydrous dioxane (10 mL), diethylvinylphosphonate (5 mmol, 0.77 ml) and tyramine (5 mmol, 0.69 g) was heated under reflux overnight. ^{31}P NMR showed that the reaction was not complete, therefore additional tyramine (0.25 g) was added and the mixture was refluxed one more day, when^{31}P NMR showed the reaction was nearly complete. The reaction mixture was cooled to room temperature and filtered to remove precipitated K_2CO_3, which was then washed with dioxane (2 x 3 mL). The liquid fractions were combined, the solvent was removed under reduced pressure, and a 250 mg portion of the crude orange-brown residue was loaded onto a neutral alumina column (5 g, 60-325 mesh, Fisher) and eluted with dichlormethane (4% v/v) in methanol to afford pure compound (60 mg). Yield 55%; *[1]H-NMR:* 1.29 t (6H, $^3J_{HH}$ 7.0 Hz, CH_3-CH_2); 1.95 t and 2.01 t (2H, $^3J_{HH}$ 7.3 Hz, -CH_2-P); 2.72 t (2H, $^3J_{HH}$ 6.4 Hz, -CH_2-Ph); 2.85 t (2H, $^3J_{HH}$ 6.7 Hz, -CH_2-NH); 2.96 t (2H, $^3J_{HH}$ 7.3 Hz,CH_2-NH); 4.07 t (4H, $^3J_{HH}$ 6.7 Hz,CH_2-O); 5.2 bs (1H, -NH); 6.88 and 7.15 (4H, arom); *[13]C-NMR:* 16.30 d (2C, $^3J_{PC}$ 5.5 Hz, CH_3-CH_2); 26.09 d (1C, $^1J_{PC}$ 139.5 Hz, CH_2-P); 34.97 s (1C, -CH_2-arom); 42.95 d (1C, $^4J_{PC}$ 3.3 Hz, -CH_2-NH); 50.76 s (1C, -CH_2-NH); 61.78 d (1C, $^2J_{PC}$ 6.7 Hz, CH_2-O); 115.57, 129.43, 129.95, 155.52 (6C, arom); *[31]P-NMR:* 30.32; *IR (neat):* 3207, 2985, 1226, 1038; Anal.calc. for $C_{14}H_{24}NO_4P$: C-55.80%, H-8.03%, N-4.65%; found C-55.56%, H-8.03%, N-5.01%.

Diethyl 2-[2-(4-methoxyphenyl)ethylamino]-3-methyl-1-butenephosphonate (**8**)

A solution of anhydrous distilled ethanol (5 mL), diethyl 3-methyl-1,2-butadienephosphonate (10 mmol, 2.0 g) and 4-methoxyphenethylamine (10 mmol, 1.5 mL) was heated under reflux overnight. The solvent was removed under reduced pressure to yield colorless crystals which were recrystallized with hexane. Yield 34%; mp 67-68^{o}C;*[1]H-NMR:* 1.02 d and 1.05 d (6H, $^3J_{HH}$ 7.0 Hz, *E*- and *Z*-CH_3-CH); 1.27 t and 1.30 t (6H,$^3J_{HH}$ 7.1 Hz, *E*- and *Z*-CH_3-CH_2); 2.54 m (1H, *E*- and *Z*-CH-CH_3); 2.76 t and 2.81 t (2H, $^3J_{HH}$ 7.0 Hz, *E*- and *Z*-CH_2-arom); 3.20 dt and 3.32 dt (2H, $^3J_{HH}$ 7.1 Hz, $^3J_{HH}$ 10.9 Hz (NH-CH_2), *E*- and *Z*-CH_2-NH); 3.51 d and 3.79 d (1H, $^2J_{PH}$ 11.1 Hz, *E*- and *Z*-CH); 3.77 s and 3.78 s (3H, *E*- and *Z*-CH_3-O); 4.02 m (4H, *E*- and *Z*- CH_2-O); 4.12 t (1H, $^3J_{HH}$ 11.9 Hz, *E*-NH) and 7.34 t (1H, $^3J_{HH}$ 6.1 Hz, *Z*-NH); 6.91 m (arom); *[13]C-NMR:* 16.33 s and 16.39 d (2C, $^4J_{PC}$ 3.3 Hz, *E*- and *Z*-CH_3-CH); 21.02 s and 21.72 s (2C, *E*- and *Z*-CH_3-CH_2); 29.42 d and 30.18 d (1C, $^3J_{PC}$ 17.6 Hz, $^3J_{PC}$ 4.8 Hz, *E*- and *Z*-CH-CH_3); 33.31 s and 36.25 s (1C, *E*- and *Z*-CH_2-arom); 43.86 s and 44.37 s (1C, *E*- and *Z*-CH_2-NH); 55.13 s and 55.20 s (1C, *E*- and *Z*-CH_3-O); 60.63 d and 61.36 d (2C, $^2J_{PC}$ 4.2 Hz, $^2J_{PC}$ 6.8 Hz, *E*- and *Z*-CH_2-O); 65.94 d and 70.79 d (1C, $^1J_{PC}$ 194.4 Hz, $^1J_{PC}$ 215.3 Hz, *E*- and *Z*-=CH-P); 113.59 – 172.71 (C-arom); 167.88 d (1C, $^2J_{PC}$ 20.9 Hz, =C-NH); *[31]P-NMR:* 26.46 and 29.28 (*E*- and *Z*-isomer);Anal.calc. for $C_{18}H_{30}NO_4P$: C-60.83%, H-8.51%, N-3.94%; found: C-60.79%, H-8.59%, N-3.93%.

Diethyl 2-[2-(3,4-dimethoxyphenyl)ethylamino]-3-methyl-1-butenephosphonate (**9**)

A solution of anhydrous distilled ethanol (8 mL), diethyl 3-methyl-1,2-butadienephosphonate (5 mmol, 1.0 g) and 3,4-dimethoxyphenethylamine (5 mmol, 0.84 ml) (Aldrich) was heated under reflux overnight. The solvent was removed under reduced pressure to yield yellowish liquid which was fractionally distilled twice under vacuum. Yield 83%; bp 179-180^{o}C/ 0.05 mm Hg; *[1]H-NMR:* 0.95 d and 0.96 d (6H, $^3J_{HH}$ 6.7 Hz, *E*- and *Z*-CH_3-CH); 1.18 t and 1.21 t (6H, $^3J_{HH}$ 6.8 Hz, *E*- and *Z*-CH_3-CH_2); 2.42 m (1H, *E*- and *Z*-CH-CH_3); 2.65 t and 2.72 t (2H, $^3J_{HH}$ 7.3 Hz, *E*- and *Z*-CH_2-arom); 3.12 dt and 3.25 dt (2H, $^3J_{HH}$ 7.0 Hz, $^3J_{HH}$ 12.2 Hz (NH-CH_2), *E*- and *Z*-CH_2-NH); 3.42 d and 3.71 d (1H, $^2J_{PH}$ 11.3 Hz, *E*- and *Z*-CH); 3.74 s, 3.75 s, 3.76 s, and 3.78 s (6H, *E*- and *Z*-CH_3-O); 3.80 – 3.90 m (4H, *E*- and *Z*- CH_2-O); 4.19 t (1H, $^3J_{HH}$ 9.8 Hz, *E*-NH) and 7.36 t (1H, $^3J_{HH}$ 9.6 Hz, *Z*-NH); 6.61 – 6.72 m (arom); *[13]C-NMR:* 16.06 s and 16.14 s (2C, $^4J_{PH}$ 3.3 Hz, $^4J_{PH}$ 4.4 Hz, *E*- and *Z*-CH_3-CH_2); 20.76 s and 21.46 s (2C, *E*- and *Z*-CH_3-CH_2); 29.18 d and 29.96 d (1C, $^3J_{PC}$ 13.6 Hz, $^3J_{PC}$ 4.4 Hz, *E*- and *Z*-CH-CH_3); 33.55 s and 36.66 s (1C, *E*- and *Z*-CH_2-arom); 43.60 s and 44.08 s (1C, *E*- and *Z*-CH_2-NH); 55.57 s, 55.66 s, and 55.73 s (2C, *E*- and *Z*-CH_3-O); 60.37 d and 61.72 d (2C, $^2J_{PC}$ 5.5 Hz, $^2J_{PC}$ 6.6 Hz, *E*- and *Z*-CH_2-O); 65.71 d and 71.63 d (1C, $^1J_{PC}$ 194.4 Hz, $^1J_{PC}$ 216.53 Hz, *E*- and *Z*-=CH-P); 111.22 – 172.50 (C-arom); 167.11 d (1C, $^2J_{PC}$ 20.9 Hz, =C-NH); *[31]P-NMR:* 28.16 and 28.82 (*E*- and *Z*-isomer); Anal.calc. for $C_{19}H_{32}NO_4P$: C-59.21%, H-8.37%, N-3.63%; found: C-59.16%, H-8.46%, N-3.62%.

Diethyl 2-(4-hydroxyphenylthio)-3-methyl-2-butenephosphonate (**10**)

A solution of diethyl 3-methyl-1,2-butadienephosphonate (10 mmol, 2.0 g), and 4-hydroxythiophenol (10mmol, 1.26 g) in anhydrous distilled dioxane (5 mL) was heated under reflux for two days. The solvent was removed under reduced pressure to yield a slightly yellow viscous liquid, which was distilled twice under vacuum. Yield 87%; bp 190-2^{o}C/0.05 mm Hg. *[1]H-NMR:* 1.17 t (6H, $^3J_{HH}$ 7.1 Hz, -CH_3-CH_2); 1.78 d (3H, $^5J_{PH}$ 4.9 Hz, *Z*-CH_3) and 1.94 d (3H, $^5J_{PH}$ 6.1 Hz, *E*-CH_3); 2.66 d (2H, $^2J_{PH}$ 20.5 Hz, –CH_2-P); 3.96 dq (4H, $^5J_{PH}$ 14.1 Hz, $^3J_{HH}$ 7.1 Hz, -CH_2-O); 6.68 d and 6.98 d (4H, $^3J_{HH}$ 5.0 Hz, arom); 8.83 bs (1H, -OH); *[13]C-NMR:* 16.24 d (2C, $^3J_{PC}$ 5.5 Hz -CH_3-CH_2); 22.05 d and 23.25 d (2C, $^4J_{PC}$ 2.1 Hz, *Z*-CH_3-C=, $^4J_{PC}$ 3.3 Hz, *E*-CH_3-C=); 30.6 d (1C, $^1J_{PC}$ 140.6 Hz, -CH_2-P); 62.059 d (2C, , $^2J_{PC}$ 6.6 Hz, -CH_2-O); 117.51 d (1C, $^2J_{PC}$ 12.1 Hz, =C-CH_2); 116.21 s, 117.59 s ,123.62 s, 129.33 s, 132.12 s, 156.7 s (C-arom) 141.23 d (1C, $^3J_{PC}$ 12.0 Hz,=C-CH_3); *[31]P-NMR:* 28.54 ; Anal.calc. for $C_{15}H_{23}O_4PS$: C-54.53%, H-7.02%, found: C-52.62%, H-7.29%.

Diethyl 2(4-methoxyphenylthio)-3-methyl-2-butenephosphonate (**11**)

A solution of anhydrous distilled acetonitrile (5 mL), diethyl 3-methyl-1,2-butadienephosphonate (10 mmol, 2.0 g) and 4-methoxybenzenethiol (10 mmol, 1.2 mL) was heated under reflux for two days. The solvent was removed under reduced pressure to yield a slightly yellow liquid which was distilled twice under vacuum. Yield 87%;bp 145-6°C/0.05 mm Hg.*[1]H-NMR:* 1.25 t (6H, $^{3}J_{HH}$ 7.3 Hz, -CH_3-CH_2); 1.90 d (3H, $^{5}J_{PH}$ 4.6 Hz, *Z*-CH_3) and 2.03 d (3H, $^{5}J_{PH}$ 6.4 Hz, *E*-CH_3); 2.74 d (2H, $^{2}J_{PH}$ 21.4 Hz, -CH_2-P); 3.72 s (3H, -CH_3-O); 4.04 m (4H, -CH_2-O); 6.71 d and 7.14 d (4H, $^{3}J_{HH}$ 8.9 Hz, arom); *^{13}C-NMR:* 16.32 d (2C, $^{3}J_{PC}$ 6.7 Hz -CH_3-CH_2); 22.03 d and 23.42 d (2C, $^{4}J_{PC}$ 2.2 Hz, *Z*-CH_3-C=, $^{4}J_{PC}$ 3.3 Hz, *E*-CH_3-C=); 31.23 d (1C, $^{1}J_{PC}$ 140.6 Hz, -CH_2-P); 55.20 s (1C, -CH_3-O); 61.59 d (2C, , $^{2}J_{PC}$ 6.6 Hz, -CH_2-O); 117.15 d (1C, $^{2}J_{PC}$ 12.1 Hz, =C-CH_2); 142.50 d (1C, $^{3}J_{PC}$12.1 Hz,=C-CH_3); 114.50 s, 126.19 s, 131.24 s, 158.41 s (C-arom); *^{31}P-NMR:* 25.71; Anal.calc. for $C_{16}H_{25}O_4PS$: C-55.80%, H-7.32%, found C-55.58%, H-7.38%.

Cytotoxicity

The MTT (methyltetrazolium; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay was used to estimate the *in vitro* toxicity of the test compounds. G361 and SK-Mel-24human melanoma cell lines used in the drug challenges were cultured in RPMI 1640 culture medium supplemented with 10% FBS and 1% PSF at 37 ºC in a humidified atmosphere of 5 % CO_2 in air. The studies were carried out in 96 well microtitre plates inoculated on day 1 with $1x10^4$ cells per well in culture medium (100 μL). Twenty-four hours after plating serial dilutions of test compounds were added and the cells were incubated for an additional 72 hours under identical culture conditions. At the end of this period the culture medium containing the test compound was removed from the wells and replaced with RPMI 1640 culture medium (100 μL) containing 0.5 mg/mL MTT. The plates were incubated for 4 hours as above, then the MTT-containing medium was removed and replaced with isopropyl alcohol (100 μL) for 30 min to dissolve any MTT crystals in the cells. The plates were read at 540 nm on a Beckman Coulter DU640 Spectrophotometer ELISA Plate Reader. The number of viable cells per well were calculated by interpolation from the individual optical density readings, which are proportional to the number of surviving cell numbers (Mosmann, 1983).

Mushroom tyrosinase assay

Oxidation of the novel dialkyl phosphonate compounds by mushroom tyrosinase was determined using a screening assay adapted from the literature (Kahn and Andrawis, 1986). Substrate concentrations on the order of 1×10^{-4}M were incubated with mushroom tyrosinase (4.04×10^{-4}g/mL) in PBS (pH 6.8) at 37 °C for 15 min in a total volume of 2 mL PBS (pH 6.8). Incubation controls included PBS (pH 6.8; negative control), tyramine and DL-tyrosine in PBS (pH 6.8; positive controls), and two non-phenolic dialkyl phosphonates, bis(methylazeridinyl) 3-methyl-1,2-butadiene phosphonate and dimethyl 2-cyclohexylidene ethane phosphonate. The reaction mixtures were cooled with ice, post incubation to stop reaction and then visualized to determine color changeand UV spectrophotometric analysis from 200-600 nm to check for presence of a chromophore in the 289-292 nm region.

***In vivo* inhibition of melanogenesis**

Animal studies were approved by the University of Alberta Animal Care Committee in accordance with the tenets of the Canadian Council on Animal Care.

Preliminary testing of model compound **3** was carried out on a single C57BL/6J black mouse (University of Alberta Animal Services). Using a literature method to test for inhibition of melanin synthesis in anagen hair, the black hair were manually plucked from a small area on the back of the mouse under light fluothane anesthesia; this process initiates new anagen growth and activates follicular melanocytes to increase tyrosinase activity (Alena *et al*, 1990). Compound **3,** dissolved in a solution of tween and P.E.G (1:1), was administered daily for 14 days by intraperitoneal (i.p.) injection at a dose of 300 mg/kg body weight.

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