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Original article

Structure activity relationship studies of natural product chemokine receptor CCR5 antagonist anibamine toward the development of novel anti prostate cancer agents

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ABSTRACT

Recent studies have indicated that the CCR5 chemokine receptor may be a potential target for treating prostate cancer. Thus, development of CCR5 antagonists may provide novel prostate cancer therapy. Anibamine, a novel pyridine quaternary alkaloid isolated from *Aniba* sp., was found to effectively compete with ^{125}I-gp120 in binding to the chemokine receptor CCR5, with an $IC_{50} = 1$ μM. Anibamine is the first natural product reported as a CCR5 antagonist, and thus provides a novel structural skeleton unique from other lead compounds that have generally been identified from high-throughput screening efforts. In order to refine the lead compound's structure and improve the therapeutic index of anibamine derivatives as potential anti prostate cancer agents, the approach of "deconstruction–reconstruction–elaboration" was applied in the structure–activity relationship studies of this work. Here, we report the design, syntheses and anti prostate cancer activities of anibamine and 17 analogues. The results from the *in vitro* and *in vivo* studies described here show that this class of compounds has potential to provide novel leads as anti prostate cancer agents.

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1. Introduction

Prostate cancer is the most common non-cutaneous solid cancer occurring among men in the USA. The most recent estimates from the American Cancer Society [1] showed that about 240,890 new cases of prostate cancer would be diagnosed in 2011, with 33,720 deaths attributable to prostate cancer in the United States alone. About one of six males in the U.S. may be afflicted with this cancer, and the risk is increased drastically for older males. Moreover, the risk of death due to metastatic prostate cancer is 1 in 36. Genetics, age, race, diet, and family history, and even lifestyle may all contribute to prostate cancer risk [2]. The treatment options for prostate cancer are surgery, chemotherapy, cryotherapy, hormonal therapy and/or radiation, but all are only beneficial at the early stages, with no significant effects after metastasis [3]. Therefore, there is a high need for treatments that will stop the metastasis and invasion of prostate cancer cells.

Historically, chronic inflammation has been believed to play a role in the development of many types of cancer in humans [4–7], including prostate cancer [2,8]. During the past decade, genetic studies have provided evidence that infection or inflammation of the prostate does contribute to the development of prostate cancer. Inflammation is thought to incite carcinogenesis by causing cell and genome damage, promoting cellular turnover, and creating a tissue microenvironment that can enhance cell replication, angiogenesis and tissue repair [9]. It is usually self-limiting and thus does not cause any harm to the body. However, sometimes the control is lost and inflammation becomes chronic leading to various diseases such as Crohn's disease, Blau syndrome and cancer, among others [10]. The inflammatory response begins when injured tissue cells release chemical signals (inflammatory mediators or cytokines), which then attract other immune cells, increase capillary permeability, and cause fever. Chemokines are a family of small cytokines that play a number of important roles in acute and chronic inflammation and also have immunoregulatory functions. These specialized cytokines attract monocytes, macrophages, neutrophils,

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and eosinophils, cells that are responsible for nonspecific inflammatory immune response [11]. Since chronic inflammation has been linked to tumors and cancer, chemokines may also play important roles in creating a microenvironment that favors or enhances tumor survival. Chemokine receptors expressed by target cells may promote numerous downstream events related to tumor angiogenesis, growth, survival and metastasis through the binding of these soluble protein ligands [4].

Expression of the inflammatory chemokine CCL5 (RANTES) by tumor cells is thought to correlate with the progression of several cancers [6,12–14]. For example, the chemokine CCL5 was shown to induce breast cancer cell migration that was mediated by the chemokine receptor CCR5. It was also shown that the expression of CCR5 and CCL5 correlated with breast cancer disease progression while a CCR5 antagonist inhibited breast tumor growth in the presence of CCL5 [15,16]. A recent investigation also showed that CCR5 is overexpressed in prostate cancer specimens [17]. A preclinical study of the CCR5 antagonist TAK-779 [18] showed that TAK-779 blocked RANTES-induced cell invasion and proliferation of prostate cancer cells [14]. Thus, development of an appropriate chemokine receptor CCR5 antagonist may provide a novel prostate cancer therapy.

CCR5, a G-protein-coupled receptor, has also been identified as an essential co-receptor for HIV-1 entry to host cells [19,20] and has thus become an attractive target for development of anti-HIV therapeutics [21–23]. A number of small molecule CCR5 antagonists developed based on leads identified through high-throughput screening, e.g., SCHC [24], aplaviroc [25], vicriviroc [26,27], maraviroc [28] and TAK-779 [18,29] (Fig. 1), have shown potent activities in blocking chemokine function and HIV entry. However, SCHC caused a modest but dose-dependent prolongation of the corrected cardiac QT interval (QTc) [27]; the vicriviroc clinical trial was stopped because the drug did not meet primary efficacy endpoints in late stage trials [30]; and development of TAK-779 as an anti-HIV-1 agent was discontinued due to its poor oral bioavailability [31]. The first CCR5 antagonist in clinical trials, aplaviroc, showed initial potent antiviral activity but, after severe hepatotoxicity occurred in several patients, its development was stopped in October 2005 [25]. In fact, to date only one drug, maraviroc, has been approved by the FDA (on August 6, 2007), although concerns were raised that maraviroc could be associated with increased risks of liver damage, lymphoma, infections and heart attack [32]. It is clear that, in order to develop new CCR5 antagonists, there is an urgent need to explore new chemical structures and templates with a wider range of structural features. Compared to high-throughput screening hits, lead compounds derived from natural products contain more diverse skeletons with wider ranges of shape and chemical features, often with specific biological activities [33,34]. Thus, natural products are desirable and useful resources for drug discovery and development.

Anibamine (1), a unique pyridine quaternary alkaloid recently isolated from the Guyanese plant *Aniba panurensis* [35,36], has been found to effectively compete with ^{125}I-gp120 for binding with the chemokine receptor CCR5 with an IC_{50} value of 1 μM. As the first reported natural product that is a CCR5 antagonist, anibamine possesses a structural skeleton that is remarkably different from all previous lead compounds, all of which were discovered through high-throughput screening studies. Recent studies also demonstrated that anibamine produced significant inhibition of prostate cancer cell proliferation at micromolar to submicromolar levels as well as suppressing adhesion and invasion of the highly metastatic M12 prostate cancer cell line [37]. These findings suggest that anibamine may serve as a new lead for development of CCR5 antagonists with potential applications in prostate cancer therapy. In this paper we explore and define the structure–activity relationships of anibamine and its analogues as anti prostate cancer agents with application of the "deconstruction–reconstruction–elaboration" approach. The results reported here will be the basis of future studies in our long-term goal to discover an optimized CCR5 antagonist.

2. Results and discussion

2.1. Molecular design

The "deconstruction–reconstruction–elaboration" approach (Fig. 2) has been successfully applied to improve the pharmacological activities of both synthetic agents and natural products [38–41]. In this case, each structural component of the lead was "removed" one at a time to test the influence of that component on CCR5 antagonism and anti prostate cancer activity. Once the essential structural components were defined, they were retained in the core structure that was then further modified to improve these activities.

2.2. Chemistry

The total synthesis of anibamine was first reported by our laboratory a few years ago [42]. Very recently, we accomplished an improved synthetic methodology with high stereo- and regioselectivity [43]. These approaches were applied in the present work for the syntheses of newly designed derivatives of anibamine to

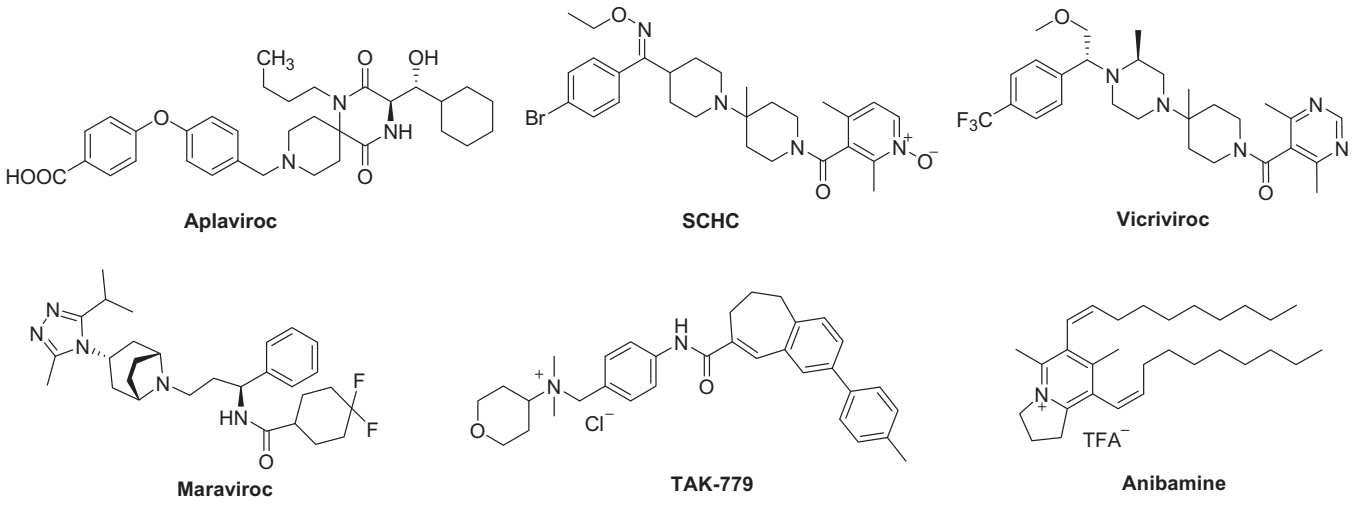


Fig. 1. Chemical structures of anibamine and some of the known CCR5 antagonists.

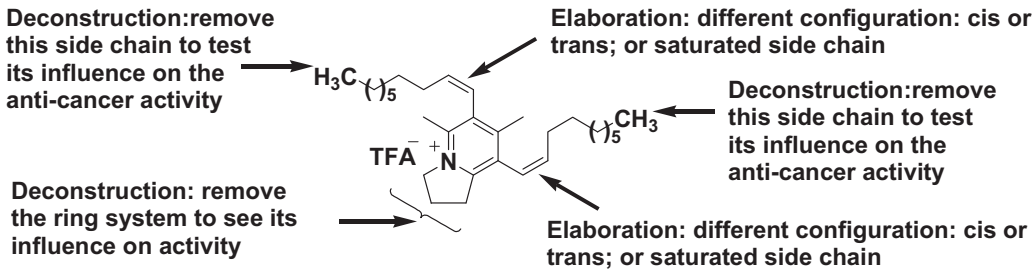


Fig. 2. Initial structural modifications of anibamine.

identify the critical pharmacophore and, ideally, a next generation lead compound for drug development.

2.2.1. Side chain deconstruction

The first deconstruction efforts were to remove the side chains at either position 3 or 5 on the core ring of the molecule. This tests the role of each side chain with respect to the anticancer activity of anibamine. The syntheses of analogues retaining the 5-position side chain are illustrated in Scheme 1. Bromination of commercially available 2-amino-4,6-dimethylpyridine in glacial acetic acid gave the desired 2-amino-5-bromo-4,6-dimethylpyridine (**1**) [44], which was diazotized under $Br_2/NaNO_2$ in 48% HBr to afford **2** [45]. The regioselective synthesis of the key intermediate 2-alkynyl-5-bromo-4,6-dimethylpyridine (**3**) was achieved by reacting **2** with a protected terminal acetylene under palladium-catalyzed Sonogashira conditions [46,47] with 76% yield. Then, following the similar scheme for the total synthesis of anibamine [43], the corresponding analogue **7** was finished with yield of 79%. In order to prepare analogues retaining the side chain at the 3-position (Scheme 2), we first condensed cyanoacetamide and acetylacetone in a potassium carbonate solution of approximately pH 9 at room temperature overnight [42]. The product **8** precipitates in 92% yield. The bromination of **8** with TBAB/P_2O_5 led to the 2-bromo-substituted intermediate **9** [48]. After hydrolysis and Hofmann rearrangement [48], the key intermediate **10** was afforded in 75% yield. Following palladium-catalyzed Sonogashira coupling reactions, reduction, diazotization and bromination, the intermediates **13** were afforded in reasonable yields. Applying a similar scheme as used for compound **7** produced analogue **16** with a satisfactory yield of 78%.

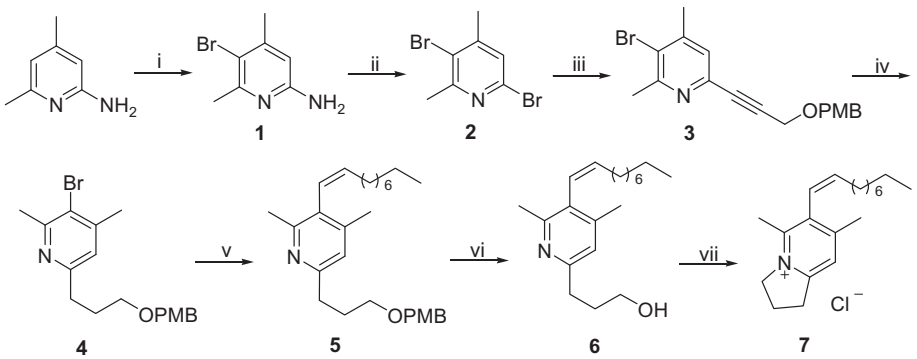
2.2.2. Core ring deconstruction

By deconstructing the rings, we aimed to clarify the contributions of anibamine's fused five-member ring; the aliphatic portion of the core ring system was thus removed in deconstruction derivatives. Based on molecular modeling and docking studies of anibamine and three other potent CCR5 antagonists [49], the positively charged nitrogenous center of the core ring system appeared to be especially important by interacting with Glu283 in the common antagonist binding locus in the CCR5 receptor. The first ring deconstruction derivative was analogue **18**. In it, a methyl group was retained at position 2 to support retention of the overall conformation, which might have been lost by removal of the fused ring. The synthetic route for **18** is shown in Scheme 3. Bromination of 2,4,6-trimethylpyridine was conducted with excess NBS, with added TFA to boost the acidity of the concentrated sulfuric acid medium [50]. The dibrominated product **17** was generated quantitatively at 50 °C. Subsequently, under Suzuki cross-coupling conditions, 3,5-di((*Z*)-dec-1-enyl)-2,4,6-trimethylpyridine (**18**) was obtained in excellent yield.

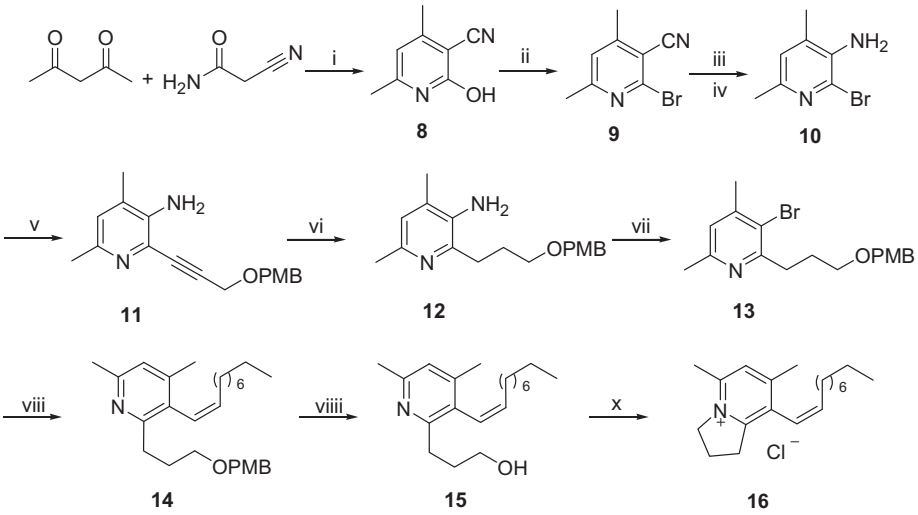
The second molecule, **21**, in the ring deconstruction set was designed to retain the count of carbon atoms (from the aliphatic ring) to minimize the change in hydrophobicity. To facilitate the synthesis, a methoxyl group was applied to cap the end of the new side chain. The synthesis scheme for **21** (Scheme 4) is nearly identical to the total synthesis of anibamine we reported earlier [43]. In order to regioselectively introduce 3-methoxypropyl at the 2-position, the key intermediate 2,3,5-tribromo-4,6-dimethylpyridine was coupled with methyl propargyl ether to afford compound 3,5-dibromo-2-(3-methoxyprop-1-ynyl)-4,6-dimethylpyridine (**19**) in 94.6% yield. Catalytic hydrogenation of the triple bond, followed by Suzuki coupling, yielded the final compound **21**.

2.2.3. Simultaneous deconstruction of core ring and side chains

In order to further elucidate the structure–activity relationship of the core ring and the side chain functionalities, analogues that simultaneously deconstructed both the core ring and side chains were also designed and synthesized. These compounds were synthesized following procedures similar to those for **7** and **16**. Synthesis of analogue **24** (Scheme 5) began with intermediate **2**,



Scheme 1. The synthesis of 5-position side chain analogues. Reagents and conditions: (i) Br_2, CH_3COOH, 68%; (ii) Br_2, $NaNO_2$, HBr (48%), 92%; (iii) $HC{\equiv}CCH_2OPMB$, CuI, $PdCl_2(PPh_3)_2$, Piperidine, 76%; (iv) H_2, PtO_2, TEA, EtOH, 86%; (v) diisopropyl (*Z*)-1-decenylboronate (1.5 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 58%; (vi) 1 N HCl, EtOH, 74%; (vii) MsCl, TEA, 79%.



Scheme 2. The synthesis of 3-position side chain analogues. Reagents and conditions: (i) K_2CO_3, H_2O, 92%; (ii) TBAB, P_2O_5, 95%; (iii) H_2SO_4, 120 °C, 75%; (iv) Br_2, NaOH, 100%; (v) $HC{\equiv}CCH_2OPMB$, CuI, $PdCl_2(PPh_3)_2$, Piperidine, 69%; (vi) H_2, PtO_2, TEA, EtOH, 77%; (vii) $CuBr_2$, $BuNO_2$, $CHBr_3$, 50%; (viii) diisopropyl (*Z*)-1-decenylboronate (1.5 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 84%; (viiii) 1N HCl, EtOH, 80.3%; (x) MsCl, TEA, 78%.

which was converted to 3-bromo-6-(3-methoxyprop-1-ynyl)-2,4-dimethylpyridine (**22**) by the Sonogashira coupling reaction using commercial available 3-methoxyprop-1-yne. Catalytic hydrogenation of the triple bond, followed by Suzuki coupling, afforded the final compound **24** in good yield.

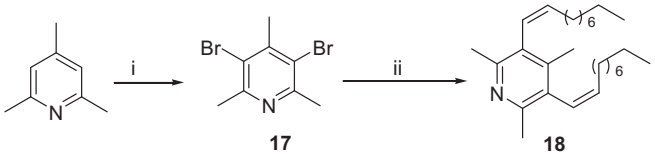
Scheme 6 describes the synthesis of analogue **28**. 2-Bromo-4,6-dimethylpyridin-3-amine (**10**) was coupled with 3-methoxyprop-1-yne under Sonogashira coupling conditions, followed by catalytic hydrogenation, diazotization and bromination, and finally Suzuki coupling, to yield **28**. Similarly, compound **30** was prepared (Scheme 7) to examine the influence of removal of both the entire aliphatic ring (retaining only a methyl vestige as in **18**) and one side chain. The procedure was very similar to that of **18**, except that the bromination reaction was carried out at room temperature.

2.2.4. Elaboration of side chains

In designing the set of compounds that elaborate the side chain, our goal was to elucidate the role of bond order and configuration in the two side chains with respect to the observed anticancer activity. In order to change the side chain alkene configuration from *cis* to *trans*, (*E*)-dec-1-enylboronic acid was used instead of diisopropyl (*Z*)-1-decenylboronate (Scheme 8). Under the same Suzuki cross-coupling reaction conditions as above, the coupled products with *E*-configuration were afforded stereoselectively in excellent yield. The unsaturated analogue, compound **34**, was prepared by reducing the double bond of **33**. Furthermore, we recently reported the regio- and stereo-selective syntheses of the (11*E*, 22*Z*) and (11*Z*, 22*E*) anibamine isomers (**35** and **36**, respectively, Fig. 3) [43].

2.2.5. Side chain deconstruction/stereochemical elaboration

Further deconstruction/elaboration products based on the anibamine main skeleton were designed to exhaust the structural variants of anibamine. Compounds **39** and **41** tested a combination of the deconstruction and elaboration illustrated above by removing one side chain in anibamine while changing the stereochemistry on the other. The chemical syntheses of compounds **39** and **41** are shown in Scheme 9. Compounds **40** and **42** were prepared by simply reducing the double bonds of **39** and **41**, respectively.



Scheme 3. The synthesis of the 2-methyl analogue (**18**). Reagents and conditions: (i) NBS, TFA, H_2SO_4, 50 °C, 100%; (ii) diisopropyl (*Z*)-1-decenylboronate (4.0 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 82%.

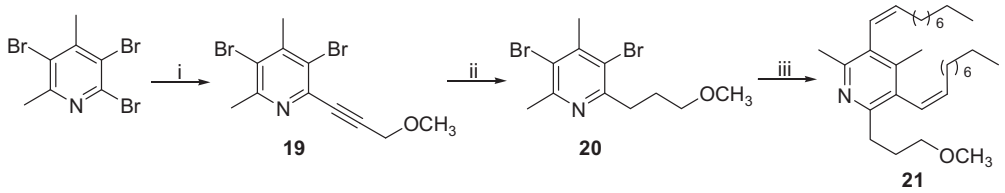
2.2.6. Elaboration of ring size

Because the positively charged nitrogen atom of anibamine is believed to be an essential feature for binding to the CCR5 receptor, we wanted to investigate how changes in the size (and resulting conformation) of the anibamine core might change its binding affinity and, further, influence anticancer activity. The ring size-modified anibamine analogues **37** and **38** (Fig. 3) were prepared following previously reported procedures, and both final compounds were obtained in good yields [51].

In summary, a series of anibamine analogues were designed following the "deconstruction–reconstruction–elaboration" approach to identify the critical pharmacophore of the natural product lead compound. A total of seventeen novel ligands that are anibamine derivates have been synthesized through multi-step chemical reactions. They all have been fully characterized by NMR, IR, MS, and HPLC (see Supporting information) prior to characterization of their biological activities, which will be described below.

2.3. Biological screening

We adopted a series of screening methods to assess the biological activity of the newly synthesized anibamine analogues as anti prostate cancer agents. First, a Ca^{2+} mobilization assay was developed to characterize their binding affinity and function against the CCR5 receptor. Similar Ca^{2+} mobilization assays have previously been applied in characterizing the binding affinity and function of other GPCR ligands [52,53]. Second, since the expression of CCL5 and CCR5 has been observed in various prostate cancer cell lines, including M12, DU145 and PC-3 [41,52,54], the anti-proliferative activities of anibamine and its analogues were also evaluated against these cell lines. Further, a basal cytotoxicity assay was conducted to evaluate the therapeutic window for these ligands to help us identify our "next generation" lead. Lastly, a prostate tumor mouse model was applied to test *in vivo* our lead's anti prostate cancer activity.



Scheme 4. The synthesis of the methoxypropyl analogue (**21**). Reagents and conditions: (i) $HC{\equiv}CCH_2OCH_3$, CuI, $PdCl_2(PPh_3)_2$, Piperidine, 95%; (ii) H_2, PtO_2, TEA, EtOH, 80%; (iii) diisopropyl (*Z*)-1-decenylboronate (4.0 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 80%.

2.3.1. Ca^{2+} mobilization assay

The inhibitory effects of CCR5 antagonists in chemokine-induced calcium ion mobilization have been demonstrated to correlate well with their affinity in radioligand competition binding assays [51–53]. In this study, anibamine and its analogues were tested for their ability to inhibit chemokine CCL5-induced calcium mobilization in MOLT-4/CCR5 cells as an indicator of their antagonism of CCR5 [51]. The results are shown in Tables 1 and 2. All of the analogues were tested first for their capacity as agonists to stimulate calcium release in the absence of CCL5. None of the anibamine analogues acted as agonists of CCR5 (data not shown), but most of the acted as antagonists at the low micromolar level. However, the core ring deconstruction analogues, **18**, **21**, **24**, **28** and **30**, had no significant antagonism towards CCR5 ($IC_{50} > 30$ μM), which suggests that the positively charged nitrogenous center is critical for receptor binding. The side chain deconstruction products, **7** and **16**, showed significantly decreased inhibition of receptor function compared to the parent compound anibamine, which further indicated that both side chains should be retained. For the compounds we synthesized elaborating the two side chains (**33**, **34**, **35** and **36**), double bond configuration (*trans*, *cis*, or saturated) showed an insignificant difference overall in their functional inhibition of the receptor as measured with this assay. Furthermore, both the six- and seven-member ring analogues of anibamine (**37** and **38**), showed relatively weaker activity compared to the parent lead. For the dual modification analogues (compounds **39** through **42**), similar to the other side chain deconstruction analogues in Table 1 (**7** and **16**), much lower receptor inhibition activity was observed compared to that of the parent natural product.

2.3.2. Proliferation of prostate cancer cell lines without stimulation of CCL5

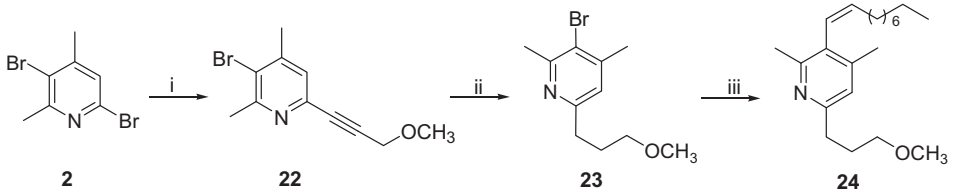
Immortalized prostate cancer cell lines provide *in vitro* models for studying this cancer. They allow the study of anti-proliferative, anti-invasive and anti-metastasis factors. For this project, three immortalized cell lines were used to study the effect of CCR5 antagonists on prostate cancer cell proliferation. The first reported CCR5 antagonist, TAK-779, was reported to inhibit proliferation and invasion of cancer cells that were pre-treated (10 ng/mL) with CCL5 [14]. Under this stimulation, TAK-779 at 500 nM showed significant inhibition of proliferation (40%–50%) for the DU145 and LNCaP cell lines. We adopted a modified protocol (without CCL5 stimulation) for anibamine and its analogues in an effort to simulate a more clinically applicable scenario as well as to avoid the possibility of non-specific stimulation arising from CCL5 interacting with other chemokine receptors normally expressed in these cancer cells.

To assess the anti-proliferative effect of these compounds, three prostate cancer cell lines were grown in the presence of anibamine and its analogues for 72 h. At that time, the colorimetric reagent WST-1 (Roche) was added and after 3 h of incubation, absorbance values were read on a FlexStation-3 (Molecular Devices) microplate reader. Most of the compounds showed dose-dependent anti-proliferative activity in all three cell lines (data not shown). The ED_{50} values of these compounds are listed in Tables 1 and 2.

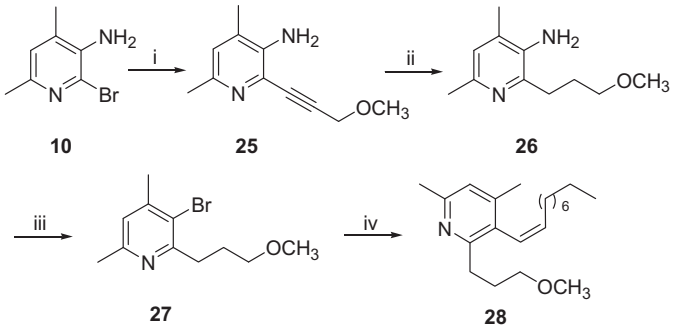
For all cell lines, the ring-deconstructed derivatives (**18**, **21**, **24**, **28** and **30**) were found to be, at best, weakly active up to the highest tested concentrations, which is consistent with the calcium mobilization assay results. On the other hand, compounds **7** and **16**, each carrying only one aliphatic side chain, showed relatively higher activity than the parent. However, considering their low affinity to CCR5 (i.e., calcium mobilization), this anti-proliferation effect on prostate cancer cells may stem from interaction with an off-target protein. The elaborated analogues (**33**, **34**, **35** and **36**, Table 1) – containing the same core but different double bond configurations on the two side chains – showed similar or higher anti-proliferation activities compared with anibamine. For the six- and seven-member ring analogues (**37** and **38**), in line with their antagonist activity, ring size did not appear to significantly influence anti-proliferation activity against the three cancer cell lines. Overall, the deconstruction/elaboration modification analogues (**39**–**42**) showed higher inhibition effects in this assay compared to the natural product lead, which again suggests off-target interactions since the Ca^{2+} mobilization assay indicates much lower activities for these compounds.

2.3.3. Basal cytotoxicity

Since some hemolysis activity has been previously reported for anibamine [36], we were interested whether the observed anti-proliferative activity of its analogues might also be associated with interactions at off-target proteins. Thus, these compounds were further evaluated for their basal cytotoxicity by adopting a Neutral Red protocol in NIH 3T3 cells [55–57]. The associated data are presented in Tables 1 and 2. For all side chain-deconstructed



Scheme 5. The synthesis of 3-((*Z*)-dec-1-enyl)-6-(3-methoxypropyl)-2,4-dimethylpyridine (**24**). Reagents and conditions: (i) $HC{\equiv}CCH_2OCH_3$, CuI, $PdCl_2(PPh_3)_2$, Piperidine, 96%; (iv) H_2, PtO_2, TEA, EtOH, 57%; (v) diisopropyl (*Z*)-1-decenylboronate (1.5 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 80%.

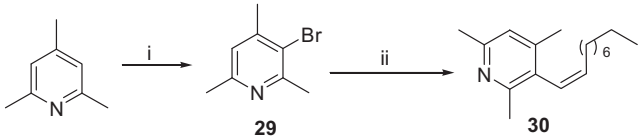


Scheme 6. The synthesis of 3-((*Z*)-dec-1-enyl)-2-(3-methoxypropyl)-4,6-dimethylpyridine (**28**). Reagents and conditions: (i) $HC{\equiv}CCH_2OCH_3$, CuI, $PdCl_2(PPh_3)_2$, Piperidine, 64%; (ii) H_2, PtO_2, TEA, EtOH, 85%; (iii) $CuBr_2$, $BuNO_2$, $CHBr_3$, 51%; (iv) diisopropyl (*Z*)-1-decenylboronate (1.5 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 72%.

analogues, including those with stereochemical elaboration (**7**, **16** and **39**–**42**), significantly higher cytotoxicities towards 3T3 cells were observed as compared with anibamine. Considering their lower receptor effects (Ca^{2+} mobilization) compared to the parent compound, clearly deconstruction of the side chain produces analogues that are non-selective. For compounds **33**–**36**, changes in the double bond configuration seemed to significantly increase their basal cytotoxicity as well, which was surprising since our preliminary molecular modeling studies suggested that the overall conformation of the molecule will not change dramatically with this elaboration [43]. Compounds **18**, **21**, **24**, **28**, and **30**, the ring deconstruction analogues, were not tested due to their low activity in both the calcium mobilization and anti-proliferation assays. Lastly, both ring size-elaboration products showed relatively low basal cytotoxicity with the six-member ring analogue **37** more tolerated with a TC_{50} similar to that of anibamine.

2.3.4. Proliferation of prostate cancer cell line under stimulation of CCL5

To further simulate experimental conditions reported in the literature, the growth of the M12 prostate cancer cell line under the stimulation of different concentrations of CCL5 was next tested. The results are summarized in Fig. 4. It is apparent that the proliferation of M12 cells show dose response to the stimulation of CCL5; under CCL5 stimulation of 30 nM, this proliferation was significantly higher than without CCL5. To verify whether the inhibitory effects of our anibamine lead compound and the two ring size-elaborated analogues (**37** and **38**) against prostate cancer cells proliferation were due to inhibition of CCL5 binding on CCR5, we tested this activity against CCL5 (10 and 30 nM) treated M12 cells. As shown in Table 2, under these conditions, all three compounds showed higher inhibition activity than without CCL5 stimulation. This seems to support the hypothesis that their effect on proliferation of M12 cells was related to their interfering in the function of the CCL5/CCR5 axial in these cells. Most interestingly, compound **38** seemed to be more sensitive to the stimulated M12 cells than compound **37**.



Scheme 7. The synthesis of compound **30**. Reagents and conditions: (i) NBS, TFA, H_2SO_4, r.t., 90%; (ii) diisopropyl (*Z*)-1-decenylboronate (4.0 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 82%.

2.3.5. In vivo study of lead compounds on tumor growth

Lastly, to assess the effect of anibamine and its analogue **38** on prostate tumor growth *in vivo*, we injected mice subcutaneously with M12 prostate cancer cells M12, followed by treatment with drug at a dose of 0.3 mg/kg (or saline) once every four days for total of sixteen days before data analysis. No apparent toxicity of the compounds to the animal, e.g., significant weight loss, was observed during the experiments. The experiment was terminated when the control group mice were lost due to their fully developed tumors. The results, in Fig. 5, show that anibamine reduced the subcutaneous growth of M12 tumors in athymic nude mice by nearly 50% at this dose, while **38** reduced the growth of the tumors, even more, by over 70% (P value < 0.05). The tumor growth reduction effects were also significantly different between anibamine and **38** (P value < 0.05). These results establish compound **38** as our new lead for future molecular design and development of a novel anti prostate cancer agent.

3. Conclusions

There is no cure for metastatic prostate cancer. Anibamine and its analogues may represent a new class of compounds that can disrupt proliferation and metastasis of prostate cancer. A series of these analogues was designed and synthesized based on the "deconstruction–reconstruction–elaboration" concept. All compounds that retained the core fused ring system acted as CCR5 antagonists in calcium mobilization assays. These analogues were also able to inhibit proliferation of prostate cancer cells *in vitro*. The core ring system appeared to be far more important than the configuration of double bonds in the side chains with respect to their overall anti-proliferation activity and cytotoxicity. One of these analogues, **38**, inhibited growth of M12 prostate tumor cells significantly in an *in vivo* model. Further structure activity relationship studies of anibamine and the new lead may help identify the next generation of anti prostate cancer agents as well as elucidate the potential role of CCL5/CCR5 axial in prostate cancer development and progression.

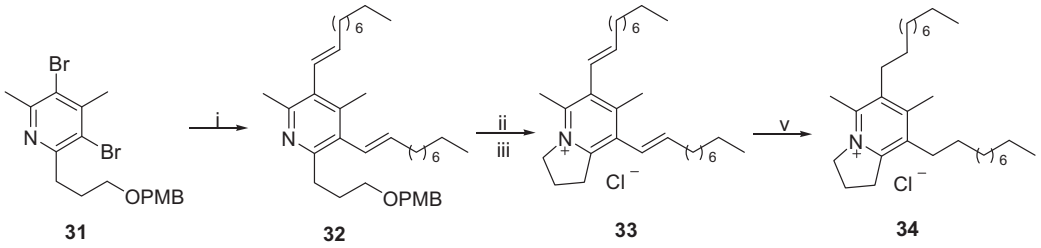
4. Experimental section

4.1. Chemistry

All reagents were used directly as obtained commercially unless otherwise noted. Melting points were determined on a Fisher-Scientific melting point apparatus and are uncorrected. ^1H NMR and ^{13}C NMR spectra were taken on a Bruker Avance III 400 MHz spectrometer with tetramethylsilane as an internal standard. ^1H spectra were collected at 400 MHz and ^{13}C spectra were collected at 100 MHz. Chemical shift values are expressed in ppm and coupling constants (J) in Hertz. Infrared spectra were obtained on a Nicolet 5ZDX FT-IR spectrometer. LC–MS was performed on a Waters Micromass QTOF-2 instrument using ESI ion source. Column chromatography was performed on silica gel (grade 60 mesh; Bodman Industries, Aston, PA). The purity of the title compounds was assessed by means of a Varian Prostar 210 HPLC instrument equipped with Prostar 325 UV/vis as the detector and working at 254 nm. A Microsorb 100-5 CN column (250 × 21.5 mm) chromatographic column was used at room temperature, a flow rate of 4.0 mL/min and run over 30 min. The eluent was acetonitrile (0.5% TFA)/water (60/40). Using the analytical conditions reported above, the retention times of the compounds range between 5.79 and 12.80 min. All the synthesized compounds were generally more than 95% pure.

4.1.1. 2-Amino-5-bromo-4,6-dimethylpyridine (**1**)

A solution of 2-amino-4,6-dimethylpyridine (1.22 g, 10 mmol) in 10 mL of glacial acetic acid under N_2 was treated with a solution of 1.6 g (10 mmol) Br_2 in 2 mL of glacial acetic acid over 15 min with



Scheme 8. The synthesis of compounds **33** and **34**. Reagents and conditions: (i) (*E*)-dec-1-enylboronic acid (4.0 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3, 87%; (ii) 1 N HCl, EtOH, 91%; (iii) MsCl, TEA, 71%; (v) H_2, 10%Pd/C, 66%.

water bath cooling to keep the reaction temperature below 20 °C. The solution became a solid mass and was allowed to stand at room temperature for 1 h. After cooling in an ice bath, the material was made alkaline with 20% cold NaOH solution. Then the mixture was extracted with CH_2Cl_2 three times, the combined organic layer was dried over anhydrous Na_2SO_4, then filtered and concentrated. The residue was purified on silica gel (Hexane–EtOAc, 2:1 to 1:1) to afford the product (1.37 g, 68%) as a yellow solid. Mp 143–144 °C. (lit. [40] 143 °C). ^{1}H NMR ($CDCl_3$) 6.24 (s, 1H), 4.32 (br, 2H), 2.50 (s, 3H), 2.28 (s, 3H).

4.1.2. 3,6-Dibromo-2,4-dimethylpyridine (**2**)

To a solution of **1** (201 mg, 1 mmol) in HBr (48%, 3 mL) at 0 °C, a solution of $NaNO_2$ (172 mg, 2.5 mmol) in H_2O (0.6 mL) was added dropwise. Then the mixture was stirred at 0–5 °C for 30 min, Br_2 (448 mg, 2.8 mmol) was added, while keeping the temperature below 5 °C. After a further 30 min, the mixture was made alkaline with 20% cold NaOH solution. Then the mixture was extracted with EtOAc three times, the combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (Hexane–EtOAc, 10:1) to afford the product (243 mg, 92%) as a pale yellow solid. Mp 62–63 °C. (lit. [41] 63 °C). ^{1}H NMR ($CDCl_3$) δ 7.20 (s, 1H), 2.65 (s, 3H), 2.37 (s, 3H).

4.1.3. 2-(3-(4-Methoxybenzyloxy)prop-1-ynyl)-5-bromo-4,6-dimethylpyridine (**3**)

A solution of **2** (263 mg, 1 mmol), 1-methoxy-4-((prop-2-ynyloxy)methyl)benzene (264 mg, 1.5 mmol), CuI (19 mg, 0.1 mmol) and piperidine (255 mg, 3 mmol) in 6 mL THF was purged by the passage of N_2 through the solution, and 35 mg (0.05 mmol) of $PdCl_2(PPh_3)_2$ was added all at once. The reaction mixture was stirred at room temperature for 3 h. Then the mixture was diluted with EtOAc, washed with water, saturated brine and dried (Na_2SO_4), filtered and concentrated. The residue was purified on silica gel (Hexane–EtOAc, 8:1) to afford the product (272 mg, 76%) as a yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2933, 2835, 1512, 1246, 1020, 818. ^{1}H NMR ($CDCl_3$) δ 7.30 (m, 2H), 7.16 (s, 1H), 6.88 (m, 2H), 4.60 (s, 2H), 4.37 (s, 2H), 3.80 (s, 3H), 2.68 (s, 3H), 2.38 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 159.4, 158.0, 147.7, 139.9, 129.8, 129.8, 129.4, 126.8, 124.2, 113.9, 113.9, 85.6, 85.0, 71.6, 57.5, 55.3, 25.8, 23.1. MS (ESI) *m*/*z*: 360.5 $(M + H)^+$.

4.1.4. 2-(3-(4-Methoxybenzyloxy)propyl)-5-bromo-4,6-dimethylpyridine (**4**)

A solution of **3** (272 mg, 0.76 mmol) in 3 mL of ethanol and 0.06 mL of triethylamine was hydrogenated over 7 mg (0.03 mmol) of PtO_2 for 3 h. The reaction mixture was filtered and concentrated. The residue was purified on silica gel (Hexane–EtOAc, 8:1) to afford the product (237 mg, 86%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2930, 2853, 1511, 1244, 1097, 1022, 818. ^{1}H NMR ($CDCl_3$) δ 7.25 (m, 2H), 6.87 (m, 2H), 6.82 (s, 1H), 4.43 (s, 2H), 3.80 (s, 3H), 3.48 (t, J = 6.4 Hz, 2H), 2.75 (m, 2H), 2.64 (s, 3H), 2.34 (s, 3H), 1.96–2.03 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 159.14, 159.06, 156.6, 147.2, 130.7, 129.2, 129.2, 122.7, 121.4, 113.8, 113.8, 72.5, 69.2, 55.3, 34.1, 29.8, 25.6, 23.2. MS (ESI) *m*/*z*: 386.3 $(M + Na)^+$.

4.1.5. 6-(3-(4-Methoxybenzyloxy)propyl)-3-((*Z*)-dec-1-enyl)-2,4-dimethylpyridine (**5**)

$Pd(OAc)_2$ (7 mg, 0.03 mmol), PPh_3 (25 mg, 0.1 mmol), and **4** (196 mg, 0.54 mmol) were stirred in toluene (1.08 mL) and aq Na_2CO_3 (0.54 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of diisopropyl (*Z*)-1-decenylboronate (216 mg, 0.81 mmol) in ethanol (0.54 mL). The solution was refluxed overnight, then diluted with EtOAc, filtered and concentrated, after which the residue was purified on silica gel (Hexane–EtOAc, 8:1) to afford the product (132 mg, 58%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2999, 2923, 2852, 1590, 1512, 1245, 1098, 1036, 819, 723. ^{1}H NMR ($CDCl_3$) δ 7.27 (m, 2H), 6.87 (m, 2H), 6.80 (s, 1H), 6.22 (d, J = 11.2 Hz, 1H), 5.69 (m, 1H), 4.44 (s, 2H), 3.80 (s, 3H), 3.51 (m, 2H), 2.78 (m, 2H), 2.39 (s, 3H), 2.14 (s, 3H), 1.98–2.06 (m, 2H), 1.76–1.81 (m, 2H), 1.19–1.34 (m, 12H), 0.86 (t, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 159.1, 158.7, 155.3, 145.5, 134.7, 130.8, 129.4, 129.2, 129.2, 125.2, 121.2, 113.8, 113.8, 72.5, 69.6, 55.3, 34.6, 31.8, 30.0, 29.4, 29.3, 29.2, 29.0, 28.7, 23.1, 22.6, 19.8, 14.1. MS (ESI) *m*/*z*: 424.7 $(M + H)^+$.

4.1.6. 3-(5-((*Z*)-Dec-1-enyl)-4,6-dimethylpyridin-2-yl)propan-1-ol (**6**)

To a solution of **5** (132 mg, 0.31 mmol) in 6 mL of EtOH was added 1 N HCl (3 mL). Then the mixture was refluxed for 3 h. After cooling, the mixture was concentrated to remove EtOH. The water layer was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified on

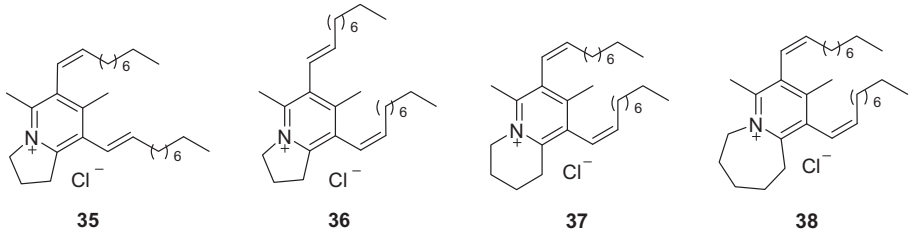
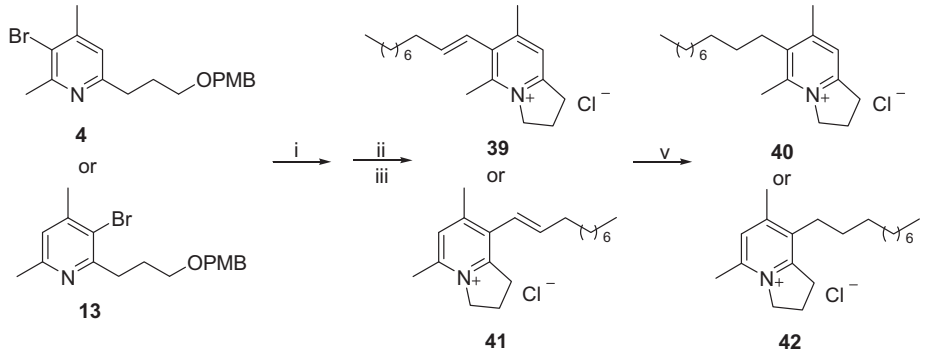


Fig. 3. Isomers of anibamine and the ring size-modified anibamine analogues.



Scheme 9. The syntheses of compounds **39**–**42**. Reagents and conditions: (i) (*E*)-dec-1-enylboronic acid (4.0 equiv), $Pd(OAc)_2$, PPh_3, Na_2CO_3; (ii) 1 N HCl, EtOH; (iii) MsCl, TEA; (v) H_2, 10%Pd/C.

silica gel (DCM–MeOH, 30:1) to give 78 mg pale yellow oil in 74% yield as the hydrochloride salt. IR (KBr, cm^{-1}) ν_{max}: 3266, 3000, 2923, 2853, 1593, 1512, 1463, 1245, 1036, 817, 729. ^1H NMR ($CDCl_3$) δ 6.85 (s, 1H), 6.20 (d, $J = 11.2$ Hz, 1H), 5.80 (m, 1H), 3.74 (m, 2H), 2.91 (m, 2H), 2.39 (s, 3H), 2.16 (s, 3H), 1.93–1.99 (m, 2H), 1.75–1.81 (m, 2H), 1.19–1.33 (m, 12H), 0.86 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 158.0, 154.9, 146.4, 135.1, 129.7, 124.7, 121.8, 62.9, 35.8, 31.9, 31.1, 29.4, 29.3, 29.2, 29.0, 28.8, 22.7, 22.6, 19.9, 14.1. MS (ESI) *m*/*z*: 304.3 $(M + H)^+$.

4.1.7. 6-Dec-1-(Z)-enyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium chloride (**7**)

Methanesulfonyl chloride (38 mg, 0.33 mmol) was added to an ice-cooled solution of **6** (50 mg, 0.16 mmol) and triethylamine (50 mg, 0.5 mmol) in 5 mL CH_2Cl_2. The resulting mixture was allowed to warm to room temperature over 1 h. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl twice, dried over Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 10:1) to afford the product (42 mg, 79%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3384, 2956, 2923, 2853, 1626, 1434, 1247, 727. ^1H NMR (CD_3OD) δ 7.70 (s, 1H), 6.31 (d, $J = 11.3$ Hz, 1H), 6.07 (dt, $J = 7.4$, 11.3 Hz, 1H), 4.71 (t, $J = 7.6$ Hz, 2H), 3.49 (t, $J = 7.4$ Hz, 2H), 2.64 (s, 3H), 2.44–2.52 (m, 2H), 2.44 (s, 3H), 1.83–1.85 (m, 2H), 1.38–1.41 (m, 2H), 1.24–1.31 (m, 10H), 0.88 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (CD_3OD) δ 158.2, 157.5, 151.0, 139.9, 136.2, 123.4, 122.9, 58.4, 33.1, 33.0, 30.5, 30.4, 30.3, 30.1, 29.8, 23.7, 21.8, 21.5, 18.1, 14.4. MS (ESI) *m*/*z*: 286.2 (M^+). HPLC: >95% pure ($t_R = 7.37$ min).

4.1.8. 2-Hydroxy-4,6-dimethylpyridine-3-carbonitrile (**8**)

Cyanoacetamide (8.4 g, 100 mmol) was dissolved in a solution of K_2CO_3 (4 g, 28.9 mmol) in water (200 mL). Then acetylacetone (10 g, 100 mmol) was added and the reaction stirred overnight at room temperature. The precipitate was filtered and washed with hexane to give the product (13.6 g, 92% yield) as a white powder. Mp 188–189 °C. (lit. [42] 188–189 °C). ^1H NMR ($CDCl_3$) δ 12.76 (br, 1H), 6.07 (s, 1H), 2.43 (s, 3H), 2.41 (s, 3H).

4.1.9. 2-Bromo-4,6-dimethylnicotinonitrile (**9**)

A solution of **8** (4.44 g, 30 mmol), TBAB (13.52 g, 42 mmol), and P_2O_5 (11.08 g, 78 mmol) in toluene (200 mL) was refluxed overnight. The toluene layer was decanted and washed with saturated solution of $NaHCO_3$ and then with water. To the oily residue left in the flask was added water and then powered $NaHCO_3$ portion-wise until there was no further evolution of gas. The mixture was extracted with CH_2Cl_2 and washed with brine. The toluene and CH_2Cl_2 solutions were combined and dried over Na_2SO_4, filtered and concentrated, followed by filtration of the product through a short pad of

Table 1
Biological screening results of anibamine and its analogues.

Compounds	$IC_{50} \pm$ SE (μM)[a]	$ED_{50} \pm$ SE (μM)[a]			$TC_{50} \pm$ SE (μM)[a]
	Ca^{2+}	M12	DU-145	PC-3	NR/3T3
Anibamine	5.4 ± 0.9	3.3 ± 0.4	1.0 ± 0.2	1.1 ± 0.1	22.7 ± 7.4
7	16.3 ± 4.2	0.8 ± 0.2	0.9 ± 0.6	0.5 ± 0.1	4.2 ± 0.4
16	9.2 ± 0.4	1.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	1.9 ± 0.1
18	>100	>30	>30	>30	n.d.[b]
21	>30	18.7 ± 3.8	>30	>30	n.d
24	>30	6.6 ± 1.0	>30	>30	n.d.
28	>30	31.7 ± 0.1	>30	>30	n.d.
30	>30	>30	>30	>30	n.d.
33	6.5 ± 1.8	2.2 ± 0.3	0.8 ± 0.1	0.6 ± 0.1	2.5 ± 0.8
34	7.8 ± 1.4	1.9 ± 0.4	1.3 ± 0.2	0.6 ± 0.1	2.8 ± 1.1
35	10.1 ± 3.9	2.2 ± 0.3	0.2 ± 0.1	0.5 ± 0.1	3.7 ± 2.3
36	9.2 ± 0.6	2.4 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	3.2 ± 1.5
37	10.0 ± 0.4	3.2 ± 0.5	0.7 ± 0.1	0.3 ± 0.1	19.0 ± 1.7
38	8.4 ± 0.9	2.6 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	7.7 ± 0.9
39	13.7 ± 2.3	0.3 ± 0.1	0.8 ± 0.5	0.2 ± 0.1	4.3 ± 0.9
40	16.4 ± 3.6	1.7 ± 0.2	1.6 ± 0.2	1.5 ± 0.4	7.0 ± 1.3
41	9.2 ± 0.4	1.3 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	1.9 ± 0.1
42	10.5 ± 2.4	0.9 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	3.2 ± 0.3

[a] Values shown were mean ± S.E. mean from at least three separate experiments performed in triplicate. The IC_{50} (concentration of CCR5 antagonist for 50% inhibition of chemokine-induced calcium ion mobilization), ED_{50} (concentration to inhibit 50 percent of cell proliferation compared with the control) and TC_{50} (toxic concentration for 50 percent of mouse embryonic fibroblast cells) values were calculated using Prism.

Table 2
Anti-proliferation assay of lead compounds against M12 cell line under CCL5 stimulation conditions.

Compound	$ED_{50} \pm SE$ (μM)[a]		
	No CCL5	10 nM CCL5	30 nM CCL5
Anibamine	3.3 ± 0.4	1.4 ± 0.2	0.8 ± 0.1
37	3.3 ± 0.5	1.5 ± 0.2	1.9 ± 0.9
38	2.6 ± 0.1	1.6 ± 0.1	0.9 ± 0.1

[a] Values shown were mean ± S.E. mean from at least three separate experiments performed in triplicate. The ED_{50} (concentration to inhibit 50 percent of cell proliferation compared with the control) values were calculated using Prism.

silica eluting with Hexane/EtOAc (2:1). The product was afforded (6.0 g, 95% yield) as a white solid. Mp 116–117 °C. (lit. [48] 116–117 °C). ^{1}H NMR: ($CDCl_3$) δ 7.10 (s, 1H), 2.57 (s, 3H), 2.54 (s, 3H).

*4.1.10. 2-Bromo-4,6-dimethylpyridin-3-amine (**10**)*

A solution of **9** (420 mg, 2 mmol) in concentrated H_2SO_4 (2 mL) was heated at 120 °C for 2 h. Then the reaction mixture was cooled to room temperature and poured into ice water. The solution was neutralized with solid $NaHCO_3$. The mixture was extracted with CH_2Cl_2, washed with brine and dried over Na_2SO_4, then filtered. Evaporation of the solvent afforded 2-bromo-4,6-dimethylpyridine-3-carboxamide (343 mg, 75%) as a white solid. Mp 179–180 °C. (lit. [48] 179–180 °C). Br_2 (2.8 g, 17.5 mmol) was added to a solution of NaOH (2.1 g, 52.5 mmol) in 21 mL of water at −5 °C. 2-bromo-4,6-dimethylpyridine-3-carboxamide (3.19 g, 14 mmol) was added in one portion. The reaction mixture was stirred at 0 °C for 1 h and then at 70 °C for 1 h. The reaction mixture was then cooled to room temperature and extracted with CH_2Cl_2; the organic layer was washed with brine, dried (Na_2SO_4), filtered and concentrated. The residue was purified on silica gel (Hexane–EtOAc, 2:1) to afford the product (2.8 g, 100%) as a pale yellow solid. Mp 61–62 °C. (lit. [48] 61–62 °C). ^{1}H NMR ($CDCl_3$) δ 6.80 (s, 1H), 3.90 (br s, 2H), 2.39 (s, 3H), 2.18 (s, 3H).

*4.1.11. 2-(3-(4-Methoxybenzyloxy)prop-1-ynyl)-4,6-dimethylpyridin-3-amine (**11**)*

A solution of **10** (400 mg, 2 mmol), 1-methoxy-4-((prop-2-ynyloxy)methyl)benzene (422 mg, 2.4 mmol), CuI (19 mg, 0.1 mmol) and piperidine (510 mg, 6 mmol) in 10 mL THF was purged by passing N_2 through the solution, and 140 mg (0.2 mmol) of $PdCl_2(PPh_3)_2$ was added all at once. The reaction mixture was stirred at room temperature for 2 d. Then, the mixture was diluted

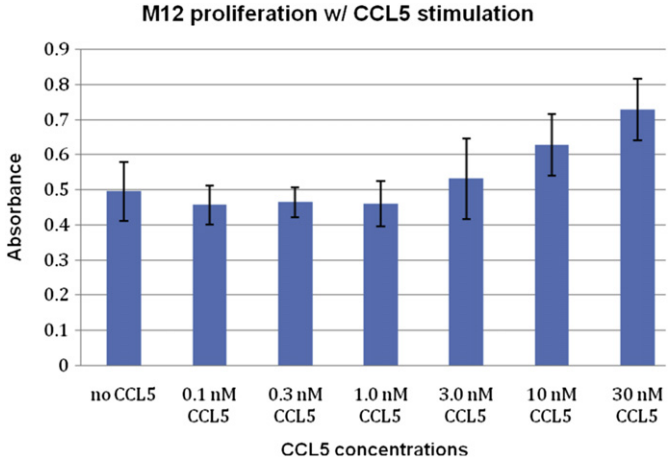


Fig. 4. CCL5 stimulation of M12 prostate cancer cell proliferation.

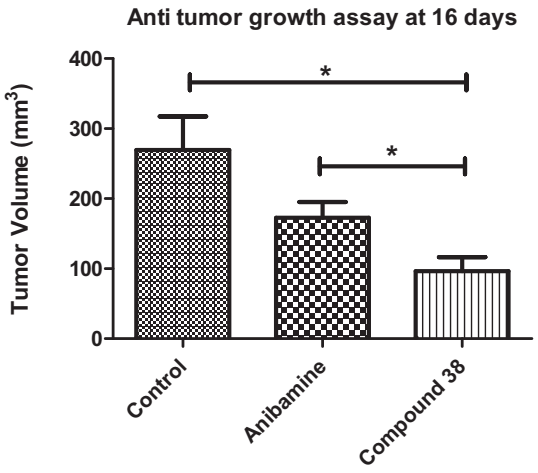


Fig. 5. *In vivo* studies of prostate tumor growth inhibition of two lead compounds. Statistical analysis was conducted by using *t*-test.

with EtOAc, washed with water, saturated brine and dried (Na_2SO_4), filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 1:1) to afford the product (410 mg, 69%) as a yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3362, 2934, 2837, 1733, 1612, 1512, 1462, 1243, 1074, 1033, 818. ^{1}H NMR (CD_3Cl_3) δ 7.30 (m, 2H), 6.88 (m, 2H), 6.84 (s, 1H), 4.61 (s, 2H), 4.45 (s, 2H), 4.04 (br s, 2H), 3.80 (s, 3H), 2.40 (s, 3H), 2.15 (s, 3H). ^{13}C NMR (CD_3Cl_3) δ 159.4, 148.1, 141.3, 131.1, 129.7, 129.7, 129.5, 126.4, 125.2, 113.9, 113.9, 90.4, 82.8, 71.4, 57.7, 55.3, 23.4, 17.0. MS (ESI) *m*/*z*: 297.2 $(M + H)^+$.

*4.1.12. 2-(3-(4-Methoxybenzyloxy)propyl)-4,6-dimethylpyridin-3-amine (**12**)*

A solution of **11** (740 mg, 2.5 mmol) in 9 mL of ethanol and 0.18 mL of triethylamine was hydrogenated over 23 mg (0.1 mmol) of PtO_2 for 3 d. The reaction mixture was filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 20:1) to afford the product (575 mg, 77%) as a brown oil. IR (KBr, cm^{-1}) ν_{max}: 3359, 2923, 2855, 1611, 1511, 1463, 1244, 1094, 1031, 818. ^{1}H NMR (CD_3Cl_3) δ 7.27 (m, 2H), 6.88 (m, 2H), 6.72 (s, 1H), 4.46 (s, 2H), 3.81 (s, 3H), 3.68 (br, 2H), 3.50 (t, J = 5.9 Hz, 2H), 2.83 (t, J = 7.3 Hz, 2H), 2.40 (s, 3H), 2.12 (s, 3H), 1.97–2.04 (m, 2H). ^{13}C NMR (CD_3Cl_3) δ 159.2, 146.3, 144.8, 136.9, 130.5, 129.3, 129.3, 129.3, 123.1, 113.8, 113.82, 72.5, 69.0, 55.3, 29.6, 28.3, 23.0, 17.2. MS (ESI) *m*/*z*: 301.2 $(M + H)^+$.

*4.1.13. 2-(3-(4-Methoxybenzyloxy)propyl)-3-bromo-4,6-dimethylpyridine (**13**)*

To a solution of **12** (575 mg, 1.92 mmol) and $CuBr_2$ (513 mg, 2.3 mmol) in bromoform (6 mL), was added dropwise $BuNO_2$ (237 mg, 2.3 mmol) at room temperature and stirred for 2 h. Then the solution was filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 6:1) to afford the product (349 mg, 50%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2930, 2853, 1511, 1244, 1033, 1021, 818. ^{1}H NMR (CD_3Cl_3) δ 7.27 (m, 2H), 6.87 (m, 2H), 6.85 (s, 1H), 4.46 (s, 2H), 3.80 (s, 3H), 3.55 (t, J = 6.6 Hz, 2H), 3.00–3.04 (m, 2H), 2.42 (s, 3H), 2.35 (s, 3H), 2.03–2.08 (m, 2H). ^{13}C NMR (CD_3Cl_3) δ 159.3, 159.1, 155.8, 147.4, 130.9, 129.2, 129.2, 123.3, 120.9, 113.7, 113.7, 72.4, 69.7, 55.3, 34.8, 28.6, 23.7, 23.3. MS (ESI) *m*/*z*: 386.3 $(M + Na)^+$.

*4.1.14. 2-(3-(4-Methoxybenzyloxy)propyl)-3-((Z)-dec-1-enyl)-4,6-dimethylpyridine (**14**)*

$Pd(OAc)_2$ (4 mg, 0.02 mmol), PPh_3 (12 mg, 0.05 mmol), and **13** (91 mg, 0.25 mmol) were stirred in toluene (0.5 mL) and aqueous Na_2CO_3 (0.25 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of diisopropyl (*Z*)-1-decenylboronate (100 mg,

0.38 mmol) in ethanol (0.25 mL). The solution was refluxed 5 h, then diluted with EtOAc, filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 4:1) to afford the product (89 mg, 84%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3000, 2923, 2852, 1590, 1512, 1245, 1098, 1036, 819, 723. ^1H NMR ($CDCl_3$) δ 7.25 (m, 2H), 6.86 (m, 2H), 6.82 (s, 1H), 6.25 (d, $J = 11.2$ Hz, 1H), 5.78 (dt, $J = 7.2$, 11.2 Hz, 1H), 4.42 (s, 2H), 3.80 (s, 3H), 3.48 (t, $J = 6.7$ Hz, 2H), 2.75 (br, 2H), 2.46 (s, 3H), 2.14 (s, 3H), 1.90–1.98 (m, 2H), 1.75–1.81 (m, 2H), 1.19–1.33 (m, 12H), 0.86 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 159.0, 158.5, 155.4, 145.6, 134.9, 131.0, 129.1, 129.1, 128.7, 124.8, 121.9, 113.7, 113.7, 72.3, 70.0, 55.3, 32.6, 31.8, 29.4, 29.4, 29.2, 29.2, 29.0, 28.8, 24.1, 22.6, 19.8, 14.1. MS (ESI) m/z: 424.7 $(M + H)^+$.

4.1.15. 3-(3-((Z)-Dec-1-enyl)-4,6-dimethylpyridin-2-yl)propan-1-ol (**15**)

To a solution of **14** (80 mg, 0.19 mmol) in 4 mL of EtOH was added 1 N HCl (2 mL). Then the mixture was refluxed for 3 h. After cooling, the mixture was concentrated to remove EtOH. The water layer was extracted with CH_2Cl_2 (3 × 10 mL) and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 30:1) to give 51 mg pale yellow oil in 80% yield as the hydrochloride salt. IR (KBr, cm^{-1}) ν_{max}: 3266, 3002, 2922, 2853, 1593, 1455, 1059, 722. ^1H NMR ($CDCl_3$) δ 6.85 (s, 1H), 6.23 (d, $J = 11.2$ Hz, 1H), 5.81 (dt, $J = 7.3$, 11.2 Hz, 1H), 3.70 (t, $J = 5.4$ Hz, 2H), 2.92 (m, 2H), 2.46 (s, 3H), 2.16 (s, 3H), 1.87–1.93 (m, 2H), 1.74–1.80 (m, 2H), 1.19–1.33 (m, 12H), 0.86 (t, $J = 6.8$ Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 157.7, 154.8, 146.6, 135.3, 129.0, 124.6, 122.2, 63.0, 33.5, 31.8, 30.2, 29.4, 29.3, 29.2, 28.9, 28.8, 23.5, 22.6, 20.0, 14.1. MS (ESI) m/z: 304.2 $(M + H)^+$.

4.1.16. 8-Dec-1-(Z)-enyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium chloride (**16**)

Methanesulfonyl chloride (32 mg, 0.28 mmol) was added to an ice-cooled solution of **15** (41 mg, 0.14 mmol) and triethylamine (42 mg, 0.42 mmol) in 5 mL CH_2Cl_2. The resulting mixture was allowed to warm to room temperature over 1 h. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl twice, dried over Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 10:1) to afford the product (34 mg, 78%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3374, 2956, 2925, 2854, 1628, 1459, 1326, 733. ^1H NMR ($CDCl_3$) δ 7.42 (s, 1H), 6.18 (d, $J = 11.4$ Hz, 1H), 6.02 (dt, $J = 7.4$, 11.4 Hz, 1H), 4.98 (t, $J = 7.5$ Hz, 2H), 3.33 (t, $J = 7.4$ Hz, 2H), 2.84 (s, 3H), 2.51–2.59 (m, 2H), 2.40 (s, 3H), 1.80–1.86 (m, 2H), 1.18–1.38 (m, 12H), 0.86 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 156.3, 156.1, 150.2, 139.1, 131.7, 127.5, 120.4, 57.2, 32.3, 31.8, 29.4, 29.3, 29.19, 29.15, 28.8, 22.6, 20.9, 20.3, 20.1, 14.1. MS (ESI) m/z: 286.2 (M^+). HPLC: >95% pure ($t_R = 7.71$ min).

4.1.17. 3,5-Dibromo-2,4,6-trimethylpyridine (**17**)

To a solution of 2,4,6-trimethylpyridine (605 mg, 5 mmol) in TFA (3 mL) were added concentrated H_2SO_4 (4.1 mL) and NBS (5.34 g, 30 mmol) and the resultant reaction was stirred at 50 °C for 2 d. The contents were poured into crushed ice and the solution was made alkaline with 10% NaOH solution. The suspension was extracted with EtOAc twice. The organic layers were combined, washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 10:1) to afford the product (988 mg, 71%) as a white crystal. Mp 81–82 °C. (lit. [50] 81.5 °C). ^1H NMR ($CDCl_3$) δ 2.62 (s, 6H), 2.60 (s, 3H).

4.1.18. 3,5-Di((Z)-dec-1-enyl)-2,4,6-trimethylpyridine (**18**)

$Pd(OAc)_2$ (6 mg, 0.03 mmol), PPh_3 (19 mg, 0.07 mmol), and **17** (55 mg, 0.2 mmol) were stirred in toluene (0.8 mL) and aq Na_2CO_3 (0.4 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of diisopropyl (*Z*)-1-decenylboronate (214 mg, 0.8 mmol) in ethanol (0.4 mL). The solution was refluxed 6 h, diluted with EtOAc, filtered and concentrated; the residue was purified on silica gel (hexane–EtOAc, 10:1) to afford the product (65 mg, 82%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3000, 2955, 2922, 2853, 1559, 1440, 722. ^1H NMR ($CDCl_3$) δ 6.25 (d, $J = 11.2$ Hz, 2H), 5.78 (dt, $J = 7.3$, 11.2 Hz, 2H), 2.40 (s, 6H), 2.05 (s, 3H), 1.75–1.81 (m, 4H), 1.18–1.33 (m, 24H), 0.86 (t, $J = 6.9$ Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 153.2, 153.2, 143.6, 134.5, 134.5, 129.2, 129.2, 125.8, 125.8, 31.9, 31.9, 29.4, 29.4, 29.3, 29.3, 29.2, 29.2, 28.9, 28.9, 28.7, 28.7, 23.1, 23.1, 22.6, 22.6, 17.4, 14.1, 14.1. MS (ESI) m/z: 398.5 $(M + H)^+$. HPLC: >95% pure ($t_R = 9.25$ min).

4.1.19. 3,5-Dibromo-2-(3-methoxyprop-1-ynyl)-4,6-dimethylpyridine (**19**)

A solution of 2,3,5-tribromo-4,6-dimethylpyridine (682 mg, 2 mmol), methyl propargyl ether (168 mg, 2.4 mmol), CuI (19 mg, 0.1 mmol) and piperidine (510 mg, 6 mmol) in 10 mL THF was purged by passing N_2 through the solution, and 140 mg (0.2 mmol) of $PdCl_2(PPh_3)_2$ was added all at once. The reaction mixture was stirred at room temperature for 1 h. Then the mixture was diluted with EtOAc, washed with water, saturated brine and dried (Na_2SO_4), filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 10:1) to afford the product (626 mg, 95%) as a pale yellow solid. Mp 41–42 °C. IR (KBr, cm^{-1}) ν_{max}: 2978, 2930, 2820, 2235, 1539, 1428, 1360, 1098, 1046, 902. ^1H NMR ($CDCl_3$) δ 4.40 (s, 2H), 3.50 (s, 3H), 2.64 (s, 3H), 2.63 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 156.5, 147.4, 140.7, 123.8, 123.0, 89.9, 84.7, 60.3, 58.0, 25.8, 24.8. MS (ESI) m/z: 332.2 $(M + H)^+$.

4.1.20. 3,5-Dibromo-2-(3-methoxypropyl)-4,6-dimethylpyridine (**20**)

A solution of **19** (104 mg, 0.31 mmol) in 5 mL of ethanol and 0.1 mL of triethylamine was hydrogenated over 3 mg (0.01 mmol) of PtO_2 for 1 h. The reaction mixture was filtered and concentrated. The residue was purified on silica gel (Hexane–EtOAc, 20:1) to afford the product (84 mg, 80%) as a pale yellow solid. Mp 45–46 °C. IR (KBr, cm^{-1}) ν_{max}: 2922, 2866, 1547, 1438, 1385, 1117, 1040, 974. ^1H NMR ($CDCl_3$) δ 3.46 (t, $J = 6.5$ Hz, 2H), 3.35 (s, 3H), 2.98 (m, 2H), 2.62 (s, 3H), 2.61 (s, 3H), 1.96–2.03 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 157.6, 155.1, 146.6, 121.4, 121.0, 72.2, 58.5, 34.6, 28.2, 25.7, 24.8. MS (ESI) m/z: 336.2 $(M + H)^+$.

4.1.21. 3,5-Di((Z)-dec-1-enyl)-2-(3-methoxypropyl)-4,6-dimethylpyridine (**21**)

$Pd(OAc)_2$ (11 mg, 0.05 mmol), PPh_3 (38 mg, 0.14 mmol), and **20** (134 mg, 0.4 mmol) were stirred in toluene (1.6 mL) and aq Na_2CO_3 (0.8 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of diisopropyl (*Z*)-1-decenylboronate (427 mg, 1.6 mmol) in ethanol (0.8 mL). The solution was refluxed 5 h, diluted with EtOAc, filtered and concentrated; the residue was purified on silica gel (hexane–EtOAc, 8:1) to afford the product (146 mg, 80%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3000, 2955, 2922, 2853, 1456, 1377, 1119, 722. ^1H NMR ($CDCl_3$) δ 6.29 (d, $J = 11.1$ Hz, 1H), 6.25 (d, $J = 11.2$ Hz, 1H), 5.78 (m, 2H), 3.42 (t, $J = 6.7$ Hz, 2H), 3.32 (s, 3H), 2.73 (br, 2H), 2.39 (s, 3H), 2.05 (s, 3H), 1.92 (m, 2H), 1.78 (m, 4H), 1.19–1.33 (m, 24H), 0.86 (t, $J = 6.8$ Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 156.3, 153.4, 143.5, 134.7, 134.4, 129.2, 128.8, 125.9, 125.4, 72.7, 58.4, 32.5, 31.9, 29.7, 29.6, 29.6, 29.4, 29.4, 29.3, 29.2, 29.0, 28.94, 28.92, 28.8, 28.7, 23.3, 22.7, 22.7, 17.5, 14.1, 14.1. MS (ESI) m/z: 456.5 $(M + H)^+$. HPLC: >95% pure ($t_R = 11.01$ min).

4.1.22. 3-Bromo-6-(3-methoxyprop-1-ynyl)-2,4-dimethylpyridine (**22**)

A solution of 3,6-dibromo-2,4-dimethylpyridine (316 mg, 1.2 mmol), methyl propargyl ether (101 mg, 1.4 mmol), CuI (11 mg, 0.06 mmol) and piperidine (306 mg, 3.6 mmol) in 8 mL THF was

purged by the passage of N_2 through the solution, and 84 mg (0.12 mmol) of $PdCl_2(PPh_3)_2$ was added all at once. The reaction mixture was stirred at room temperature for 1 h. Then the mixture was diluted with EtOAc, washed with water, saturated brine and dried (Na_2SO_4), filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 6:1) to afford the product (290 mg, 96%) as a pale yellow solid. IR (KBr, cm^{-1}) ν_{max}: 2926, 2821, 1575, 1446, 1358, 1099, 1020, 907. ^1H NMR ($CDCl_3$) δ 7.17(s, 1H), 4.32(s, 2H), 3.45(s, 3H), 2.67 (s, 3H), 2.38 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 158.0, 147.7, 139.9, 126.7, 124.2, 85.3, 85.0, 60.3, 57.9, 25.7, 23.1. MS (ESI) *m/z*: 254.5 $(M + H)^+$.

4.1.23. 3-Bromo-6-(3-methoxypropyl)-2,4-dimethylpyridine (**23**)

A solution of **22** (290 mg, 1.15 mmol) in 6 mL of ethanol and 0.12 mL of triethylamine was hydrogenated over 10 mg (0.04 mmol) of PtO_2 for 1.5 h. The reaction mixture was filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 6:1) to afford the product (168 mg, 57%) as a pale yellow solid. IR (KBr, cm^{-1}) ν_{max}: 2922, 2866, 1586, 1448, 1389, 1116, 1021. ^1H NMR ($CDCl_3$) δ 6.86 (s, 1H), 3.41 (t, $J = 6.4$ Hz, 2H), 3.34 (s, 3H), 2.73 (m, 2H), 2.65 (s, 3H), 2.36 (s, 3H), 1.96 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 159.0, 156.7, 147.3, 122.7, 121.5, 72.0, 58.6, 34.0, 29.7, 25.6, 23.2. MS (ESI) *m/z*: 258.5 $(M + H)^+$.

4.1.24. 3-((Z)-Dec-1-enyl)-6-(3-methoxypropyl)-2,4-dimethylpyridine (**24**)

$Pd(OAc)_2$ (4 mg, 0.02 mmol), PPh_3 (14 mg, 0.05 mmol), and **23** (77 mg, 0.3 mmol) were stirred in toluene (0.6 mL) and aqueous Na_2CO_3 (0.3 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of diisopropyl (*Z*)-1-decenylboronate (120 mg, 0.45 mmol) in ethanol (0.3 mL). The solution was refluxed overnight, then diluted with EtOAc, filtered and concentrated, the residue was purified on silica gel (Hexane–EtOAc, 8:1) to afford the product (76 mg, 80%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2954, 2922, 2853, 1591, 1458, 1383, 1119, 723. ^1H NMR ($CDCl_3$) δ 6.83 (s, 1H), 6.22 (d, $J = 11.2$ Hz, 1H), 5.78 (dt, $J = 7.3, 11.2$ Hz, 1H), 3.43 (t, $J = 6.5$ Hz, 2H), 3.35 (s, 3H), 2.76 (t, $J = 7.3$ Hz, 2H), 2.40 (s, 3H), 2.15 (s, 3H), 1.98 (m, 2H), 1.29 (m, 2H), 1.19–1.34 (m, 12H), 0.86 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 158.7, 155.4, 145.6, 134.7, 129.4, 125.2, 121.2, 72.3, 58.5, 34.5, 31.8, 29.9, 29.4, 29.3, 29.2, 29.0, 28.7, 23.1, 22.6, 19.9, 14.1. MS (ESI) *m/z*: 318.1 $(M + H)^+$. HPLC: >95% pure ($t_R = 6.75$ min).

4.1.25. 2-(3-Methoxyprop-1-ynyl)-4,6-dimethylpyridin-3-amine (**25**)

A solution of **10** (400 mg, 2 mmol), methyl propargyl ether (168 mg, 2.4 mmol), CuI (19 mg, 0.1 mmol) and piperidine (510 mg, 6 mmol) in 10 mL THF was purged by passing N_2 through the solution, and 140 mg (0.2 mmol) of $PdCl_2(PPh_3)_2$ was added all at once. The reaction mixture was stirred at room temperature overnight. Then the mixture was diluted with EtOAc, washed with water, saturated brine and dried (Na_2SO_4), filtered and concentrated. The residue was purified on silica gel (Hexane–EtOAc, 1:2) to afford the product (242 mg, 64%) as a yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3344, 3199, 2925, 2820, 1620, 1462, 1105, 1094. ^1H NMR ($CDCl_3$) δ 6.83 (s, 1H), 4.40 (s, 2H), 4.05 (br, 2H), 3.46 (s, 3H), 2.40 (s, 3H), 2.15 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 148.1, 141.2, 131.1, 126.3, 125.2, 90.0, 82.9, 60.5, 57.8, 23.4, 17.0. MS (ESI) *m/z*: 190.9 $(M + H)^+$.

4.1.26. 2-(3-Methoxypropyl)-4,6-dimethylpyridin-3-amine (**26**)

A solution of **25** (300 mg, 1.58 mmol) in 7.5 mL of ethanol and 0.16 mL of triethylamine was hydrogenated over 14 mg (0.06 mmol) of PtO_2 overnight. The reaction mixture was filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 20:1) to afford the product (261 mg, 85%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3358, 2921, 2868, 1633, 1463, 1111. ^1H NMR ($CDCl_3$) δ 6.72 (s, 1H), 3.67 (br, 2H), 3.41 (t, $J = 5.92$ Hz, 2H), 3.36 (s, 3H), 2.79 (t, $J = 7.24$ Hz, 2H), 2.38 (s, 3H), 2.14 (s, 3H), 1.94–2.01 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 146.7, 145.1, 136.7, 130.9, 122.9, 71.7, 58.4, 29.8, 28.2, 23.3, 17.2. MS (ESI) *m/z*: 195.1 $(M + H)^+$.

4.1.27. 3-Bromo-2-(3-methoxypropyl)-4,6-dimethylpyridine (**27**)

To a solution of **26** (219 mg, 1.13 mmol) and $CuBr_2$ (303 mg, 1.36 mmol) in bromoform (4 mL), was added dropwise $BuNO_2$ (140 mg, 1.36 mmol) at room temperature, followed by stirring for 2 h. Then the solution was filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 6:1) to afford the product (148 mg, 51%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2922, 2867, 1586, 1446, 1117, 1020. ^1H NMR ($CDCl_3$) δ 6.86 (s, 1H), 3.47 (t, $J = 6.6$ Hz, 2H), 3.35 (s, 3H), 3.01 (m, 2H), 2.43 (s, 3H), 2.35 (s, 3H), 2.01 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 159.3, 155.8, 147.5, 123.3, 120.9, 72.4, 58.5, 34.7, 28.5, 23.7, 23.3. MS (ESI) *m/z*: 258.5 $(M + H)^+$.

4.1.28. 3-((Z)-Dec-1-enyl)-2-(3-methoxypropyl)-4,6-dimethylpyridine (**28**)

$Pd(OAc)_2$ (4 mg, 0.02 mmol), PPh_3 (12 mg, 0.05 mmol), and **27** (64 mg, 0.25 mmol) were stirred in toluene (0.5 mL) and aqueous Na_2CO_3 (0.25 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of diisopropyl (*Z*)-1-decenylboronate (100 mg, 0.38 mmol) in ethanol (0.25 mL). The solution was refluxed 5 h, then diluted with EtOAc, filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 4:1) to afford the product (57 mg, 72%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2955, 2922, 2853, 1590, 1454, 1373, 1118, 723. ^1H NMR ($CDCl_3$) δ 6.82 (s, 1H), 6.26 (d, $J = 11.2$ Hz, 1H), 5.79 (dt, $J = 7.2, 11.2$ Hz, 1H), 3.40 (t, $J = 6.7$ Hz, 2H), 3.32 (s, 3H), 2.74 (m, 2H), 2.47 (s, 3H), 2.14 (s, 3H), 1.91 (m, 2H), 1.79 (m, 2H), 1.20–1.34 (m, 12H), 0.86 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 158.4, 155.4, 145.6, 134.9, 128.7, 124.7, 121.9, 72.6, 58.4, 32.4, 31.8, 29.4, 29.3, 29.2, 29.0, 28.9, 28.8, 24.1, 22.6, 19.8, 14.0. MS (ESI) *m/z*: 318.1 $(M + H)^+$. HPLC: >98% pure ($t_R = 6.13$ min).

4.1.29. 3-Bromo-2,4,6-trimethylpyridine (**29**)

To a solution of 2,4,6-trimethylpyridine (1.21 g, 10 mmol) in TFA (2 mL) were added conc. H_2SO_4 (2.7 mL) and NBS (5.34 g, 30 mmol) and the resultant reaction was stirred at room temperature for 2 d. The contents were poured into crushed ice and the solution was made alkaline with 10% NaOH. The suspension was extracted with EtOAc twice. The organic layers were combined, washed with brine, dried with Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 4:1) to afford the product (1.79 g, 90%) as a pale yellow oil. ^1H NMR ($CDCl_3$) δ 6.86 (s, 1H), 2.64 (s, 3H), 2.44 (s, 3H), 2.35 (s, 3H). (lit. [50])

4.1.30. 3-((Z)-Dec-1-enyl)-2,4,6-trimethylpyridine (**30**)

$Pd(OAc)_2$ (3 mg, 0.01 mmol), PPh_3 (10 mg, 0.04 mmol), and **29** (40 mg, 0.2 mmol) were stirred in toluene (0.4 mL) and aqueous Na_2CO_3 (0.2 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of diisopropyl (*Z*)-1-decenylboronate (80 mg, 0.3 mmol) in ethanol (0.2 mL). The solution was refluxed overnight, then diluted with EtOAc, filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 8:1) to afford the product (46 mg, 88%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3000, 2955, 2922, 2853, 1592, 1456, 1381, 721. ^1H NMR ($CDCl_3$) δ 6.82 (s, 1H), 6.22 (d, $J = 11.2$ Hz, 1H), 5.78 (d t, $J = 7.3, 11.2$ Hz, 1H), 2.46 (s, 3H), 2.40 (s, 3H), 2.14 (s, 3H), 1.79 (m, 2H), 1.19–1.33 (m, 12H), 0.86 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 155.3, 145.6, 134.8, 129.1, 125.2, 121.8, 31.8, 29.4, 29.3, 29.2, 29.0, 28.7, 24.0, 23.1, 22.6, 19.8, 14.1. MS (ESI) *m/z*: 260.2 $(M + H)^+$. HPLC: >98% pure ($t_R = 5.79$ min).

4.1.31. 6,8-Didecyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium chloride (**34**)

Hydrogenation was conducted on **33** (15 mg, 0.027 mmol) and 10% Pd/C (2 mg, 10% by weight) in MeOH (2 mL) under 50 psi H_2 for 6 h. The

mixture was filtered through celite to remove Pd/C and concentrated to remove MeOH to afford the product (10 mg, 66% yield) as a pale yellow oil. ^{1}H NMR (CD_3CN) δ 4.57 (m, 2H), 2.75 (m, 2H), 2.72 (m, 2H), 2,58 (s, 3H), 2.40 (s, 3H), 2.35 (m, 4H), 1.44 (m, 6H), 1.28 (m, 26 H), 0.89 (m, 6H). (lit. [47]) HPLC: >95% pure (t_R = 12.80 min).

4.1.32. 6-Dec-1-(E)-enyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium chloride (**39**)

$Pd(OAc)_2$ (5 mg, 0.02 mmol), PPh_3 (18 mg, 0.07 mmol), and **4** (138 mg, 0.38 mmol) were stirred in toluene (0.76 mL) and aqueous Na_2CO_3 (0.38 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of (*E*)-dec-1-enylboronic acid (105 mg, 0.57 mmol) in ethanol (0.38 mL). The solution was refluxed overnight, then diluted with EtOAc, filtered and concentrated. The residue was purified on silica gel (hexane–EtOAc, 8:1) to afford the product 6-(3-(4-methoxybenzyloxy)propyl)-3-((*E*)-dec-1-enyl)-2,4-dimethylpyridine (116 mg, 72%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2923, 2852, 1511, 1245, 1099, 1035, 819. ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 2H), 6.87 (m, 2H), 6.79 (s, 1H), 6.26 (d, J = 16.1 Hz, 1H), 5.69 (dt, J = 6.9, 16.1 Hz, 1H), 4.44 (s, 2H), 3.80 (s, 3H), 3.50 (t, J = 6.5 Hz, 2H), 2.76 (t, J = 6.9 Hz, 2H), 2.48 (s, 3H), 2.24 (s, 3H), 2.24 (m, 2H), 2.01 (m, 2H), 1.47 (m, 2H), 1.26–1.38 (m, 10H), 0.89 (t, J = 6.6 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 159.1, 158.2, 155.3, 137.1, 131.6, 130.8, 130.4, 129.2, 129.2, 125.4, 121.9, 113.8, 113.8, 72.5, 69.6, 55.3, 34.5, 33.4, 31.9, 30.0, 29.5, 29.4, 29.3, 29.2, 23.6, 22.7, 20.4, 14.1. MS (ESI) m/z: 424.7 $(M + H)^+$.

To a solution of 6-(3-(4-methoxybenzyloxy)propyl)-3-((*E*)-dec-1-enyl)-2,4-dimethylpyridine (205 mg, 0.48 mmol) in 8 mL of EtOH was added 1 N HCl (4 mL). Then the mixture was refluxed for 3 h. After cooling, the mixture was concentrated to remove EtOH. The water layer was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 30:1) to give 3-(5-(*E*)-dec-1-enyl)-4,6-dimethylpyridine as 156 mg pale yellow oil in 95% yield as the hydrochloride salt. IR (KBr, cm^{-1}) ν_{max}: 3261, 2954, 2923, 2853, 1626, 1486, 1336, 1233, 1040, 977. ^{1}H NMR (4$CDCl_3$) δ 6.84 (s, 1H), 6.24 (d, J = 16.2 Hz, 1H), 5.70 (dt, J = 6.9, 16.2 Hz, 1H), 3.73 (t, J = 5.6 Hz, 2H), 2.89 (t, J = 6.0 Hz, 2H), 2.47 (s, 3H), 2.25 (s, 3H), 2.24 (m, 2H), 1.94 (m, 2H), 1.24–1.51 (m, 12H), 0.89 (t, J = 6.5 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 157.5, 154.8, 146.0, 137.5, 130.7, 125.0, 122.4, 62.8, 35.7, 33.4, 31.9, 31.2, 29.4, 29.3, 29.3, 29.2, 23.2, 22.7, 20.4, 14.1. MS (ESI) m/z: 304.3 $(M + H)^+$. Methanesulfonyl chloride (105 mg, 0.92 mmol) was added to an ice-cooled solution of 3-(5-((*E*)-dec-1-enyl)-4,6-dimethylpyridine (156 mg, 0.46 mmol) and triethylamine (139 mg, 1.38 mmol) in 8 mL CH_2Cl_2. The resulting mixture was allowed to warm to room temperature over 1 h. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl twice, dried over Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 10:1) to afford the product (78 mg, 53%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3385, 2956, 2923, 2853, 1626, 1486, 1336, 1233, 1040, 977. ^{1}H NMR ($CDCl_3$) δ 7.57 (s, 1H), 6.21 (d, J = 16.2 Hz, 1H), 5.86 (dt, J = 6.9, 16.2 Hz, 1H), 5.00 (t, J = 7.5 Hz, 2H), 3.58 (t, J = 7.9 Hz, 2H), 2.82 (s, 3H), 2.58 (m, 2H), 2.46 (s, 3H), 2.29 (m, 2H), 1.49 (m, 2H), 1.29–1.37 (m, 10H), 0.89 (t, J = 6.6 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 155.9, 155.2, 149.8, 142.2, 136.2, 122.5, 121.6, 57.9, 33.2, 32.4, 31.8, 29.3, 29.1, 29.1, 28.7, 22.6, 21.7, 21.0, 18.7, 14.0. MS (ESI) m/z: 286.2 (M^+). HPLC: >97% pure (t_R = 7.29 min).

4.1.33. 6-Decyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium chloride (**40**)

Hydrogenation was conducted on **39** (25 mg, 0.08 mmol) and Pd/C (2.5 mg, 10% by weight) in MeOH (2 mL) under 50 psi H_2 for 6 h. The mixture was filtered through celite to remove Pd/C and concentrated to remove MeOH to afford the product (18 mg, 72% yield) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2922, 2853, 2666, 2654, 1690, 1633, 1199, 1122, 1040. ^{1}H NMR (CD_3CN) δ 7.53 (s, 1H), 4.58 (m, 2H), 3.37 (m, 2H), 2.74 (m, 2H), 2.62 (s, 3H), 2.51 (s, 3H), 2.38 (m, 2H), 1.44 (m, 4 H), 1.28 (m, 12 H) 0.89 (m, 3H). ^{13}C NMR (CD_3CN) δ 155.9, 154.5, 149.8, 137.8, 122.3, 56.9, 48.5, 38.5, 31.4, 31.3, 29.0, 28.72, 28.67, 28.1, 27.9, 22.1, 20.2, 19.7, 16.1, 13.1. MS (ESI) m/z: 289.0 (M^+).

4.1.34. 8-Dec-1-(E)-enyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium chloride (**41**)

$Pd(OAc)_2$ (4 mg, 0.02 mmol), PPh_3 (12 mg, 0.05 mmol), and **13** (91 mg, 0.25 mmol) were stirred in toluene (0.5 mL) and aqueous Na_2CO_3 (0.25 mL, 2 M) under N_2 for 0.5 h. To this solution was added a solution of (*E*)-dec-1-enylboronic acid (69 mg, 0.38 mmol) in ethanol (0.25 mL). The solution was refluxed overnight, then diluted with EtOAc, filtered and concentrated; the residue was purified on silica gel (hexane–EtOAc, 4:1) to afford the product 2-(3-(4-methoxybenzyloxy)propyl)-3-((*E*)-dec-1-enyl)-4,6-dimethylpyridine (91 mg, 86%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2953, 2923, 2852, 1590, 1512, 1454, 1245, 1098, 1036, 969. ^{1}H NMR ($CDCl_3$) δ 7.25 (m, 2H), 6.86 (m, 2H), 6.80 (s, 1H), 6.29 (d, J = 16.1 Hz, 1H), 5.65 (dt, J = 6.9, 16.1 Hz, 1H), 4.43 (s, 2H), 3.80 (s, 3H), 3.49 (t, J = 6.7 Hz, 2H), 2.83 (m, 2H), 2.44 (s, 3H), 2.22 (s, 3H), 2.19 (m, 2H), 1.96 (m, 2H), 1.47 (m, 2H), 1.28–1.36 (m, 10H), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 159.0, 158.5, 154.9, 145.3, 137.1, 130.9, 130.0, 129.1, 129.1, 125.1, 122.4, 113.7, 113.7, 72.3, 70.0, 55.3, 33.4, 32.6, 31.9, 29.6, 29.5, 29.4, 29.3, 29.3, 24.0, 22.7, 20.4, 14.1. MS (ESI) m/z: 424.7 $(M + H)^+$.

To a solution of 2-(3-(4-methoxybenzyloxy)propyl)-3-((*E*)-dec-1-enyl)-4,6-dimethylpyridine (80 mg, 0.19 mmol) in 6 mL of EtOH was added 1 N HCl (3 mL). Then the mixture was refluxed for 3 h. After cooling, the mixture was concentrated to remove EtOH. The water layer was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 30:1) to give 3-(3-((*E*)-dec-1-enyl)-4,6-dimethylpyridin-2-yl)propan-1-ol as 55 mg pale yellow oil in 85% yield as the hydrochloride salt. IR (KBr, cm^{-1}) ν_{max}: 3262, 2954, 2923, 2853, 1593, 1455, 1246, 1036, 970. ^{1}H NMR ($CDCl_3$) δ 6.83 (s, 1H), 6.26 (d, J = 16.1 Hz, 1H), 5.65 (dt, J = 6.9, 16.1 Hz, 1H), 3.69 (t, J = 5.6 Hz, 2H), 2.99 (m, 2H), 2.44 (s, 3H), 2.23 (m, 2H), 2.23 (s, 3H), 1.92 (m, 2H), 1.47 (m, 2H), 1,29–1.38 (m, 10H), 0.89 (t, J = 6.6 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 157.7, 154.2, 146.2, 137.6, 130.4, 124.8, 122.6, 62.8, 33.4, 33.3, 31.9, 30.6, 29.4, 29.3, 29.3, 29.2, 23.4, 22.7, 20.5, 14.1. MS (ESI) m/z: 304.3 $(M + H)^+$.

Methanesulfonyl chloride (34 mg, 0.15 mmol) was added to an ice-cooled solution of 3-(3-((*E*)-dec-1-enyl)-4,6-dimethylpyridin-2-yl)propan-1-ol (51 mg, 0.15 mmol) and triethylamine (45 mg, 0.45 mmol) in 6 mL CH_2Cl_2. The resulting mixture was allowed to warm to room temperature over 1 h. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl twice, dried over Na_2SO_4, filtered and concentrated. The residue was purified on silica gel (DCM–MeOH, 10:1) to afford the product (39 mg, 82%) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 3445, 2956, 2923, 2853, 1626, 1490, 1464, 1340, 1039, 978. ^{1}H NMR ($CDCl_3$) δ 7.41 (s, 1H), 6.25 (d, J = 16.2 Hz, 1H), 6.05 (dt, J = 6.8, 16.2 Hz, 1H), 5.05 (t, J = 7.7 Hz, 2H), 3.51 (t, J = 7.8 Hz, 2H), 2.87 (s, 3H), 2.57 (m, 2H), 2.47 (s, 3H), 2.28 (m, 2H), 1.49 (m, 2H), 1.24–1.37 (m, 10H), 0.89 (t, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 155.5, 155.0, 149.5, 142.1, 132.3, 127.9, 121.2, 57.3, 33.5, 33.0, 31.8, 29.3, 29.21, 29.17, 28.8, 22.6, 21.4, 20.7, 20.3, 14.1. MS (ESI) m/z: 286.2 (M^+). HPLC: >99% pure (t_R = 6.75 min).

4.1.35. 8-Decyl-5,7-dimethyl-2,3-dihydro-1H-indolizinium chloride (**42**)

Hydrogenation was conducted on **41** (29 mg, 0.09 mmol) and Pd/C (3 mg, 10% by weight) in MeOH (3 mL) under 50 psi H_2 for 6 h.

The mixture was filtered through celite to remove Pd/C and concentrated to remove MeOH to afford the product (21 mg, 72% yield) as a pale yellow oil. IR (KBr, cm^{-1}) ν_{max}: 2924, 2855, 2663, 2647, 1690, 1632, 1199, 1122, 1041. ^1H NMR (CD_3CN) δ 7.43 (s, 1H), 4.54 (m, 2H), 3.39 (m, 2H), 2.69 (m, 2H), 2.60 (s, 3H), 2.48 (s, 3H), 2.41 (m 2H), 1.49 (m, 2H), 1.40 (m, 2H) 1.29 (m, 12 H) 0.89 (m, 3H). ^{13}C NMR (CD_3CN) δ 156.0, 154.5, 149.8, 135.0, 127.4, 56.1, 31.3, 30.8, 29.0, 28.9, 28.71, 28.66, 28.5, 27.6, 22.6, 22.1, 20.0, 18.4, 18.3, 13.1. MS (ESI) m/z: 289.2 (M^+).

4.2. Calcium mobilization assay

MOLT-4/CCR5 cells were plated in black 96-well plates with transparent bottoms (Greiner Bio-one) at 100,000 cells per well in 50:1 HBSS:HEPES assay buffer. They were incubated for 1 h at 37 °C and 5% CO_2 with control buffer or varying concentration of antagonist for a total volume of 130 μL per well. Cells were then incubated with 50 μL of Fluo-4-AM loading buffer (40 μL 2 μM Fluo-4 dye, 100 μL 2.5 mM probenecid, in 5 mL assay buffer) for an additional hour. Then 20 μL 200 nM RANTES solution in assay buffer or assay buffer alone were added to the wells right before changes in Ca^{2+} concentration were monitored by RFU for 90 s using a microplate reader (FlexStation-3, Molecular Devices). Peak values were obtained using SoftMaxPro software (Molecular Devices) and non-linear regression curves were generated using Prism (GraphPad) to calculate IC_{50} values.

4.3. Anti-proliferation assay

All cell lines, PC-3, DU-145 and M12, were incubated at 37 °C in the presence of 5% CO_2. RPMI 1640 serum free media (GIBCO Invitrogen) containing 1% L-glutamine, 0.1% ITS (insulin, 5 μg/mL; transferrin, 5 μg/mL; and selenium, 5 μg/mL; Collaborative Research, Bedford) and 0.1% gentamicin was used to cultivate all cells. M12 cells were first incubated in media with 5% fetal bovine serum (FBS); after 24 h serum free media was added with 0.01% epidermal growth factor (EGF). DU-145 and PC-3 cell lines were incubated in media containing 10% FBS at all times. Prostate cancer tumor cells (PC-3, M12, or DU-145) were plated into 96 well plates (BD Falcon, VWR) at a concentration of 1000 cells per well. Each cell line was plated in its respective serum-containing media for a total concentration of 100 μL per well. After 24 h, various concentrations of drugs in a 50 μL PBS solution were added to the cells. Control cells were given 50 μL of PBS. 72 h after incubation with the drug, the serum containing media was replaced with 100 μL of a 9:1 solution of serum free media and WST-1 (Roche). After 3 h of incubation with WST-1, the absorbance of each well was measured by a microplate reader (FlexStation-3). Absorbance values were obtained using the SoftMaxPro software and non-linear regression curves were generated using Prism to calculate IC_{50} values.

4.4. Basal cytotoxicity assay

NIH-3T3 cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, with high-glucose, L-glutamate, and sodium pyruvate; Invitrogen) supplemented with 10% new born calf serum (NBCS, invitrogen) and 1% penicillin:streptomycin. For the NRU protocol, cells, mouse embryonic fibroblast cells (NIH-3T3) were plated into 96 well plates (Costar, Corning) at a concentration of 2000 cells/well/100 μL. Plates were incubated at 37.5 °C, 5% CO_2 for 24 h. At that point, media was discarded from the plates and 50 μL of fresh culture media was added to the wells. Plates were then treated with 50 μL of compounds at various concentrations in a dilution media made up of DMEM with 1% penicillin:streptomycin. Control wells were given 50 μL of the dilution media. After 48 h of incubation, media was removed from the plates, each well was washed with 200 μL of Hank's Buffered Salt Solution (HBSS, with calcium and magnesium, Invitrogen) and the rinsing solution was removed from the plates. To each well, 200 μL of 25 μg/mL of neutral red (NR, 0.33% solution in DPBS; Sigma) in DMEM containing 5% NBCS and 1% penicillin:streptomycin was added and plates were incubated for 3 ± 0.1 h. After incubation, NR media was removed from the plates and each well was washed with 200 μL of HBSS. The washing solution was decanted from the plates and 100 μL of a solution containing 50% ethanol, 49% H_2O, and 1% glacial acetic acid was added. Plates were shaken rapidly for 20 min while being protected from light. Once removed from the shaker, plates were allowed to sit for 5 min and absorbance at 540 nM was measured by a microplate reader (FlexStation-3). Absorbance values were obtained using SoftMaxPro software and TC_{50} values were calculated using non-linear regression curves on Prism.

4.5. In vivo M12 tumor growth inhibition of two leads

Mice (each group of ten, either control group or drug group) were injected subcutaneously with 2×10^6 M12 cells. When tumors reached a measurable size of 33 mm^3, each mouse was injected in the tail vein with 0.3 mg/kg of the anibamine or compound **38** every four days over a sixteen days period. The controls were injected with saline. At the time of necropsy, tumor size was established by gross tumor mass and estimated tumor volume and analyzed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.07.049.

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