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Novel benzimidazole phosphonates as potential inhibitors of protein prenylation

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ABSTRACT

Benzimidazole carboxyphosphonates and bisphosphonates have been prepared and evaluated for their activity as inhibitors of protein prenylation or isoprenoid biosynthesis. The nature of the phosphonate head group was found to dictate enzyme specificity. The lead carboxyphosphonate inhibits geranylgeranyl transferase II while its corresponding bisphosphonate analogue potently inhibits farnesyl diphosphate synthase. The most active inhibitors effectively disrupted protein prenylation in human multiple myeloma cells.

The enzyme geranylgeranyl transferase II (GGTase II) is a member of the protein prenyltransferase family that includes farnesyl transferase (FTase) and GGTase I.[1] GGTase II is responsible for the post-translational modification of the Rab family of small GTPases. Rabs regulate intracellular trafficking events, facilitating vesicle budding, motility, docking, and fusion.[2] Mutant forms of Rabs unable to be geranylgeranylated have been shown to be mislocalized and nonfunctional.[3] Agents that disrupt Rab geranylgeranylation, either via direct inhibition of GGTase II[4] or by diminishing the synthesis of the isoprenoid substrate geranylgeranyl diphosphate (GGDP),[5–9] induce multiple myeloma cell death by disrupting monoclonal protein trafficking and inducing the unfolded protein response pathway.[10,11] The development of inhibitors of Rab geranylgeranylation is therefore of relevance from a therapeutic perspective for multiple myeloma and other malignancies characterized by abnormal protein secretion.[12,13]

Risedronate, minodronate, and zoledronic acid are all farnesyl diphosphate synthase (FDPS) inhibitors, with IC_{50}'s of 10 nM, 3 nM, and 3 nM, respectively.[14] These agents are used clinically for osteoporosis and other bone disorders.[15] A carboxyphosphonate analogue of the bisphosphonate risedronate (**1**), 3-PEHPC (**2**) (Fig. 1), was unexpectedly found to inhibit GGTase II and disrupt Rab prenylation.[16,17] 3-PEHPC is a specific inhibitor of GGTase II, although its potency is low (reported IC_{50} range from ~32 to ~600 μM),[17–19] and high concentrations (> 5 mM) are required to yield cellular effects in myeloma cells.[10] While synthesis of several PEHPC analogues has been reported,[4] no increase in potency has been achieved. More promising is an analogue of the bisphosphonate minodronate (**3**), 3-IPEHPC (**4**), which is 25-fold more potent than 3-PEHPC as a GGTase II inhibitor.[18] The (+)-enantiomer of 3-IPEHPC **4** is ~60-fold more active than the (−)-enantiomer, but also appears to weakly inhibit another enzyme in the isoprenoid biosynthetic pathway, possibly geranylgeranyl diphosphate synthase (GGDPS).[18,19,20] Finally, a carboxyphosphonate analogue (**6**) of the bisphosphonate zoledronic acid (**5**), has been reported to disrupt Rab prenylation in cells at concentrations as low as 25 μM.[21] While an IC_{50} for this compound in an *in vitro* enzyme assay has not been reported, the authors did report an IC_{50} of 360 μM for a cell-based FRET assay which detects membrane anchorage of lipid-modified proteins.[21]

Recently, a number of other imidazole derivatives have been reported, including compounds modified at the alpha position of the carboxyphosphonates (e.g. **7**)[21] but still N-substituted, and others where the polar head group has been C-linked (e.g. **8**).[22] Some of these compounds have activity in the 10 micromolar range for GGTase II inhibition in HeLa cells,[22,23] but compounds with activity at clinically more attractive levels have proven elusive.

Given the activity of various nitrogen heterocycle bearing the carboxyphosphonate head group, we have explored the synthesis of novel benzimidazole carboxyphosphonates as potential GGTase II inhibitors.

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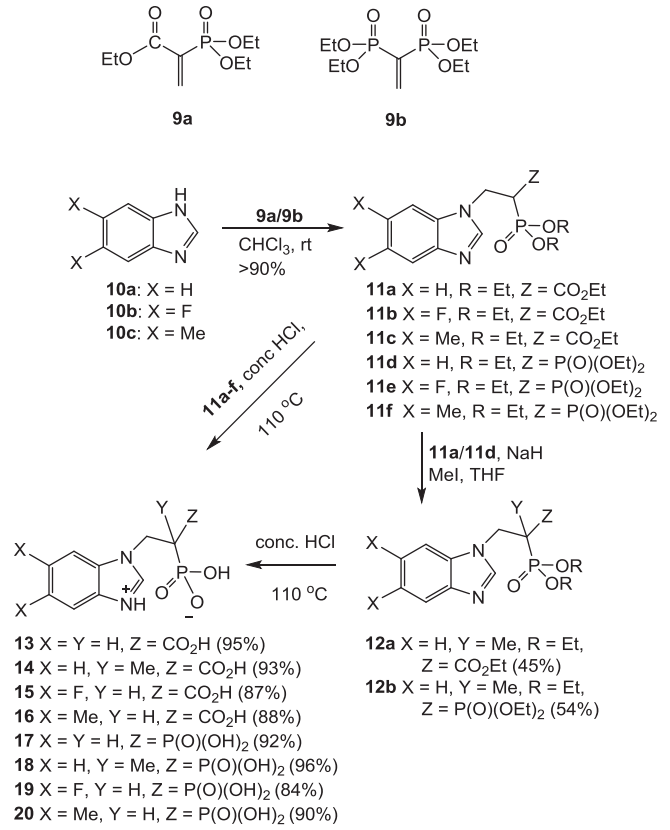
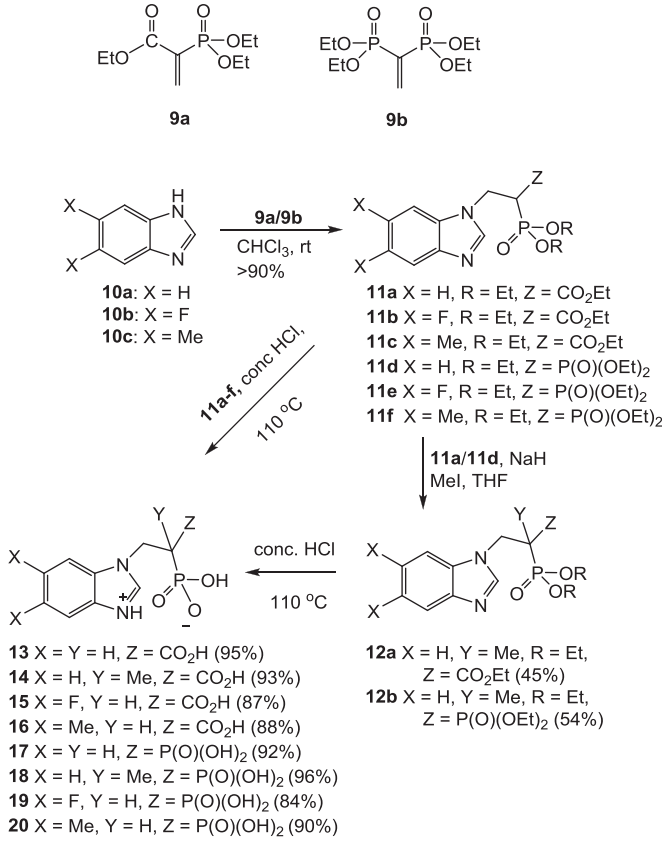


Fig. 1. Bisphosphonates and carboxyphosphonates with activity against enzymes involved in isoprenoid synthesis or protein prenylation.



Scheme 1. Benzimidazole carboxyphosphonates and bisphosphonates.

In addition, we have prepared the corresponding bisphosphonates and assessed their activity as inhibitors of FDPS and other key enzymes in isoprenoid biosynthesis. In this paper we report the synthesis and biological activity of these new compounds.

Several benzimidazole-based carboxyphosphonates (compounds **11a–c**, Scheme 1) have been synthesized by conjugate addition of the benzimidazoles **10a–c** to the vinyl carboxyphosphonate **9a**[24] through a procedure analogous to that used to prepare imidazole derivatives.[21,22] The corresponding bisphosphonates **11d–f** have been prepared following a parallel reaction sequence with the vinyl bisphosphonate **9b**.[9,25] Flash chromatography was employed if required to purify the esters using a silica column and 5% ethanol in dichloromethane, but then they were carried to the next step as quickly as possible because they undergo a retro-Michael reaction even when stored in a freezer. Two of the benzimidazole-based esters, compounds **11a** and **11d**, were methylated at the α-position through reaction with NaH and methyl iodide, to give the products **12a** and **12b** in modest yields. It is probable that the methylation step was achieved only in modest yields due to the retro-Michel reaction. In each case a substantial amount of N-methylbenzimidazole was isolated, which unexpectedly had a similar R_f value as the product carboxyphosphonate. Finally, all eight of the esters, compounds **11a–f** and **12a–b** were cleaved to the parent acids by treatment with concentrated (~12 M) hydrochloric acid at 110 °C. Experimental details and spectra can be found in the supplemental information.

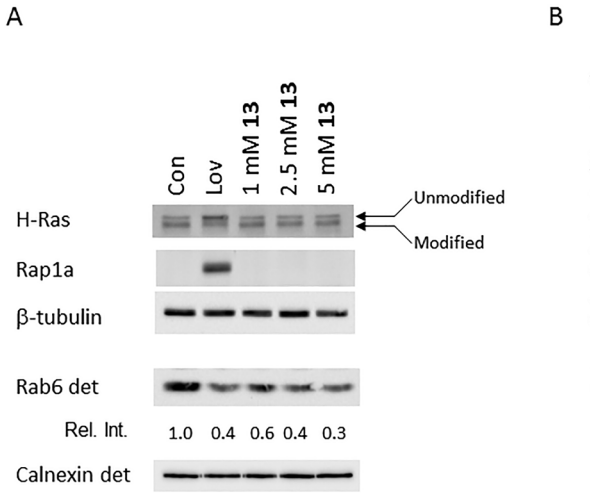
The eight new benzimidazoles **13–20** were tested for their ability to impair protein prenylation in both *in vitro* enzyme assays and cell-based assays. Of the four carboxyphosphonates, only compounds **13** and **14** were found to have cellular activity (Table 1). As shown in Fig. 2, the pattern of activity for compound **13** is consistent with GGTase II inhibition: while compound **13** did not cause disruption of H-Ras (a representative farnesylated protein) or Rap1a (a representative substrate for GGTase I) prenylation, it did cause both a decrease in membrane-bound Rab6 levels as well as an increase in intracellular light chain levels, both of which are markers for disruption of Rab geranylgeranylation.[10] Lovastatin, an HMG-CoA reductase inhibitor, was included as a positive control, as it disrupts all prenylation.[10] Consistent with the cellular data, the enzymatic data revealed that compound **13** inhibits GGTase II in a range similar to other reported GGTase II inhibitors,[4,17–19] but does not inhibit GGTase I or the synthases. Compound **13** also inhibited FTase in the enzyme assay, but there was no evidence that this inhibition was potent enough to disrupt protein farnesylation in cells (Fig. 2A). Modification of this core structure at the α-carbon diminished activity (compound **14**) while modification at the 5 and 6 positions (compounds **15** and **16**) on the benzimidazole completely abrogated the inhibitory activity.

Analysis of the benzimidazole bisphosphonates revealed that compound **17** is a potent FDPS inhibitor. As shown in Fig. 3, compound **17** globally disrupts protein prenylation, as evidenced by accumulation of unmodified Ras and Rap1a as well as intracellular lambda light chain. The enzyme assays demonstrated that compound **17** is a selective inhibitor of FDPS, with ~850-fold selectivity for FDPS compared to GGDPS and no appreciable activity against any of the prenyltransferases (Table 1). Compound **19**, the difluoro-substituted analogue also displayed cellular activity, albeit at a 20-fold higher concentration than the parent compound (Table 1). Consistent with the cellular data, the enzymatic assay data revealed that compound **19** is a less potent inhibitor of FDPS than the parent compound **17**. In addition, compound **19** lacked specificity, with weak FTase, GGTase I and GGTase II inhibitory activity *in vitro* (Table 1). Cellular activity of compound **19** was observed at a concentration 20-fold higher than that of compound **17** (data not shown). While the remaining two bisphosphonates (**18** and **20**) did inhibit FDPS in the *in vitro* enzyme assay, no detectable impairment of protein prenylation was observed in the cellular assays (data not shown). Interestingly, compound **20** was noted to inhibit GGTase II but enhance GGTase I activity in enzyme assays

Table 1
Summary of bioassay data for the novel benzimidazole phosphonates.

Compound	GGTase II (IC_{50}) (μM)	GGTase I (IC_{50}) (μM)	FTase (IC_{50}) (μM)	FDPS (IC_{50}) (μM)	GGDPS (IC_{50}) (μM)	Selectivity for FDPS relative to GGDPS	Cellular LEC[1]
13	198 ± 86	> 1000	125 ± 49	> 100	> 100	–	1 mM
14	810 ± 214	> 1000	> 1000	> 100	> 100	–	5 mM
15	> 1000	145 ± 32	450 ± 70	> 100	> 100	–	NA at ≤5 mM
16	> 1000	> 1000	> 1000	> 100	> 100	–	NA at ≤5 mM
17	> 1000	> 1000	830 ± 50	0.024 ± 0.007	20.5 ± 4.3	854	25 μM
18	> 1000	> 1000	> 1000	1.00 ± 0.11	> 100	> 100	NA at ≤1 mM
19	738 ± 112	122 ± 13	340 ± 40	0.15 ± 0.015	87 ± 22	580	0.5 mM
20	534 ± 133	> 1000*	> 1000	0.69 ± 0.29	41 ± 29	59	NA at ≤1 mM

[1]Cellular LEC (lowest effective concentration) for FDPS inhibitors is defined as the lowest concentration for which an unmodified Rap1a band is visible in the immunoblot and a statistically significant increase in intracellular lambda light chain is observed in the ELISA. Cellular LEC for GGTase II inhibitors is defined as the lowest concentration for which there is a statistically significant increase in intracellular lambda light chains and a ≥20% decrease in Rab6 levels in the detergent fraction as assessed by immunoblot analysis.
*A concentration-dependent increase in GGTase I activity was observed with enzyme activity of 560% relative to control at 1000 μM.
Abbreviation: NA, no activity.



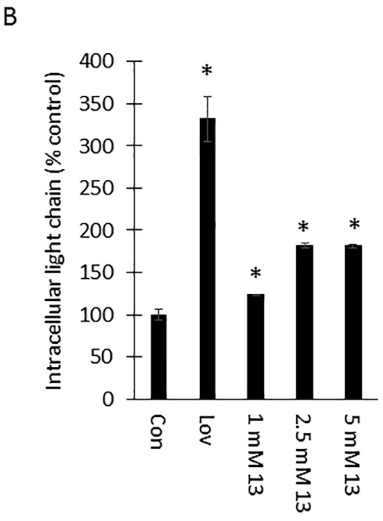
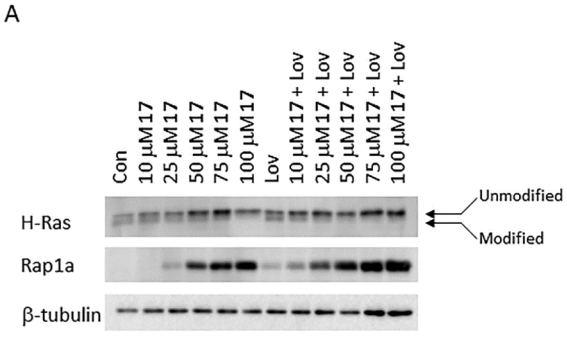


Fig. 2. Cellular activity of compound **13** is consistent with GGTase II inhibition. RPMI-8226 cells were incubated for 48 h in the presence or absence of lovastatin (10 μM, Lov) or varying concentrations of compound **13**. A) Cells were lysed using RIPA buffer to generate whole cell lysate or with Triton X-114 to generate a detergent (membrane) fraction and immunoblot analysis was performed for H-Ras, Rap1a (antibody detects only unmodified protein), and Rab6. β-Tubulin and calnexin were used as loading controls for the whole cell lysate and detergent fractions, respectively. Densitometric analysis of Rab6 (normalized to calnexin) for the treated cells normalized to untreated (control) cells is shown as relative intensity (Rel. Int.). The gels are representative of three independent studies. B) Intracellular lambda light chain concentrations were determined via ELISA. Data are expressed as percentage of control (mean ± SD, n = 3). The * denotes $p < 0.05$ per *t*-test and compares treated cells to untreated control cells.



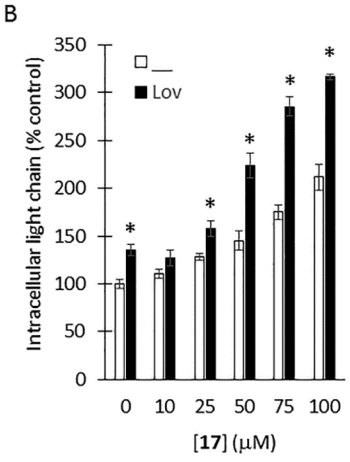


Fig. 3. Cellular activity of compound **17** is enhanced upon co-incubation with lovastatin. RPMI-8226 cells were incubated for 48 h in the presence or absence of lovastatin (0.5 μM, Lov) or varying concentrations of compound **17**. A) Immunoblot analysis of H-Ras, Rap1a (antibody detects only unmodified protein) and β-tubulin (as a loading control) was performed. B) Intracellular lambda light chain concentrations were determined via ELISA. Data are expressed as percentage of control (mean ± SD, n = 3). The * denotes statistical significance as determined by t-testing comparing treatments with and without lovastatin.

(Table 1). As previous studies have demonstrated enhanced activity of FDPS inhibitors when used in combination with HMG CoA reductase inhibitors,[26–28] combination studies were performed. As shown in Fig. 3, a low concentration of lovastatin (0.5 μM), which by itself only minimally impairs protein prenylation, enhanced the impairment of protein prenylation induced by compound **17**.

In summary, these studies are the first to utilize a benzimidazole carboxyphosphonate core in the development of GGTase II inhibitors. The most potent of this series, compound **13**, has enzymatic and cellular activity that is comparable to other reported GGTase II inhibitors. However, the addition of substituents at either the α-carbon position or at the 5 and 6 positions does not enhance the potency. The nature of the

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phosphonate head group dictates enzyme specificity such that the carboxyphosphonate **13** is a GGTase II inhibitor while the bisphosphonate version **17** is a FDPS inhibitor that has enzymatic and cellular activity that is comparable to clinically utilized FDPS inhibitors. Further structure-function work is needed to better understand the interactions between these benzimidazole phosphonates and the isoprenoid enzymes, particularly as several of the bisphosphonate derivatives displayed a lack of specificity. It is likely that factors such as isoprenoid pool sizes, flux through the isoprenoid biosynthetic pathway and hierarchical preservation of prenylation play roles in determining the impact of these inhibitors in cells. The mechanism underlying the observed increase in GGTase I activity induced by compound **20** needs to be further explored. GGTase II remains a challenging enzyme for which to develop inhibitors that are sufficiently potent to be of clinical relevance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data (representative experimental procedures, NMR spectra, and bioassay protocols) to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126757.

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