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Author manuscript

Bioorg Med Chem Lett. Author manuscript; available in PMC 2020 October 01.

Published in final edited form as:

Bioorg Med Chem Lett. 2019 October 01; 29(19): 126633. doi:10.1016/j.bmcl.2019.126633.

ω-Hydroxy isoprenoid bisphosphonates as linkable GGDPS inhibitors

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Abstract

The enzyme geranylgeranyl diphosphate synthase (GGDPS) is a potential therapeutic target for multiple myeloma. Malignant plasma cells produce and secrete large amounts of monoclonal protein, and inhibition of GGDPS results in disruption of protein geranylgeranylation which in turn impairs intracellular protein trafficking. Our previous work has demonstrated that some isoprenoid triazole bisphosphonates are potent and selective inhibitors of GGDPS. To explore the possibility of selective delivery of such compounds to plasma cells, new analogues with an ω-hydroxy group have been synthesized and examined for their enzymatic and cellular activity. These studies demonstrate that incorporation of the ω-hydroxy group minimally impairs GGDPS inhibitory activity. Furthermore conjugation of one of the novel ω-hydroxy GGDPS inhibitors to hyaluronic acid resulted in enhanced cellular activity. These results will allow future studies to focus on the *in vivo* biodistribution of HA-conjugated GGDPS inhibitors.

Graphical Abstract

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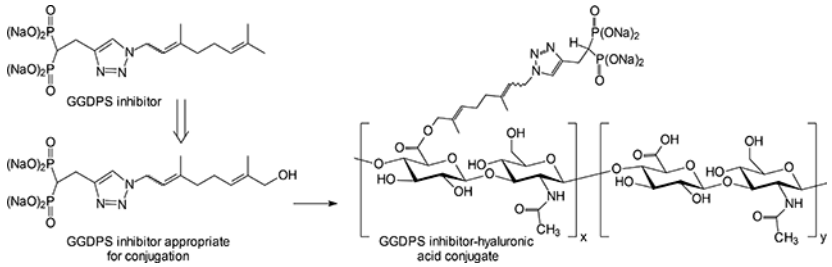

Keywords

GGDP synthase; inhibition; isoprenoid biosynthesis; triazole; bisphosphonate

Geranylgeranyl diphosphate (**1**, GGDP, Figure 1) represents an important branch point in isoprenoid biosynthetic pathways. This intermediate is used in plants to afford a tremendous variety of cyclic diterpenoids,[1] and in mammals it is used primarily for post-translational modification of proteins.[2] Among the proteins modified by reaction with GGDP are those in the Ras superfamily of small GTPases such as the Rho proteins which play roles in cancer cell migration and metastasis,[3] and the Rab proteins which are essential for intracellular trafficking processes.[4]

Isoprenoid biosynthesis in humans already is targeted by at least two families of blockbuster drugs, the statins and the nitrogenous bisphosphonates. Statins target the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) which is the rate-limiting step in the mevalonate pathway to higher isoprenoids, and these drugs are widely used as cholesterol-lowering agents and to prevent cardiovascular disease.[5] The nitrogenous bisphosphonates target the later enzyme farnesyl diphosphate synthase (FDPS), and are widely used to treat osteoporosis and other diseases of the bone.[6] *In vitro*, both classes of drugs can exert anti-cancer activities as a consequence of disruption of protein prenylation, particularly geranylgeranylation.[7] These drugs are not ideal in the setting of systemic anti-cancer therapy however, because standard doses of statins do not alter protein prenylation[8–9] and the nitrogenous bisphosphonates in clinical use do not have sufficient systemic exposure.[10]

For some time there has been interest in identification of compounds that directly inhibit the enzyme geranylgeranyl diphosphate synthase (GGDPS)[11–12] to disrupt geranylgeranylation more specifically.[11, 13] We and others have focused on the development of GGDPS inhibitors as anti-myeloma agents because disruption of Rab geranylgeranylation results in impairment of monoclonal protein trafficking within myeloma cells, leading to ER stress and cell death.[12, 14–15] In this context, we have reported the preparation and biological activity of a number of compounds with significant activity against this enzyme. The first was digeranyl bisphosphonate (**2**, DGBP),[16] which showed an IC_{50} of ~260 nM for GGDPS and good selectivity over the related enzyme farnesyl diphosphate synthase,[17] but cellular activity only at high concentrations (~10 μM). Crystallographic studies indicated that DGBP's V-shaped structure occupied the enzyme's active site, with the phosphonates complexed to magnesium ions and the two isoprenoid side chains occupying the FPP and

GGPP channels.[18] More recently we have focused on monoalkyl compounds assembled through click chemistry and incorporating a triazole ring system. These efforts first yielded the inhibitor **3**, which shows enzyme activity at 2.2 μM and cellular activity ~1 μM.[19] Surprisingly, as a mixture of olefin isomers the homologue **4** showed an IC_{50} of 45 nM against GGDPS, high specificity for GGDPS over FDPS, and cellular activity at concentrations as low as 30 nM in multiple myeloma cells.[20–21] After preparation of the individual isomers **5** and **6**, and methylation of the alpha position, bioassays revealed these compounds had IC_{50} values of 125 and 86 nM respectively, and cellular activity at 20 and 25 nM levels.[21]

The potency of these GGDPS inhibitors in both enzyme and cell assays is relevant from a therapeutic perspective. Preclinical studies of compounds **4–6** have shown systemic distribution, prolonged half-lives and metabolic stability,[22–23] all of which are important drug-like features. The dose-limiting toxicity for these compounds has been hepatic in nature[22–23] and it would be desirable to enhance their anti-myeloma activity by optimizing drug delivery to the target organs of interest and thereby minimize off-target effects. A prodrug approach might help in this regard,[24–27] but it may be preferable to utilize GGDPS inhibitors that can be conjugated to other agents. Hyaluronic acid (HA), a non-sulfated glycosaminoglycan, can be readily optimized for delivery of varied cargoes including small molecule chemotherapeutic agents,[28–30] and has thus far found clinical use in ophthalmology, rheumatology and wound healing applications.[31–32] Furthermore, work done with fluorescent dyes has revealed that conjugation of the dye to HA can lead to improved tumor uptake relative to surrounding tissue and alter biodistribution profiles.[33] HA is the native ligand for CD44 and multiple studies have demonstrated that HA can target both to CD44-overexpressed solid tumors[34–36] and to cells of hematological malignancies[37] including myeloma cells.[38] As an initial foray into this area, we describe the synthesis and biological activity of two ω-hydroxy triazole bisphosphonates. Furthermore, we demonstrate that the ω-OH modification allows linkage to HA via ester formation and report the cellular activity of the first HA-GGDPS inhibitor conjugate.

As an initial test of this strategy, the first target chosen was the triazole bisphosphonate **15** (Scheme I) which was viewed as reasonably accessible. The synthesis of this compound started with selenium dioxide catalyzed allylic oxidation of commercially available geranyl acetate (**7**).[39] While oxidation provided a mixture of the desired alcohol and the corresponding aldehyde, treatment with $NaBH_4$ to reduce that aldehyde increased the yield of the desired alcohol **8** to an acceptable level. After protection of the free alcohol **8** as the TBS ether **9**, base catalyzed hydrolysis of the acetate afforded compound **10**.[40] Conversion to the bromide **11**[41] was accomplished in near-quantitative yield by reaction with PBr_3. Reaction of the bromide **11** with sodium azide proceeded cleanly, but gave the allylic azide as a mixture of E and Z isomers due to a well known [3,3] sigmatropic rearrangement.[42] The azide then was allowed to react with the acetylene **13**[43] to afford the TBS protected triazole, which was immediately carried to acidic hydrolysis to afford the desired alcohol **14**. Standard McKenna hydrolysis[44] of the tetraethyl ester **14** by treatment with TMSBr followed by NaOH provided the tetra-sodium salt **15**. Based on the ^1H NMR spectrum of this product, the E/Z ratio was found to be ~2:1 in favor of the E isomer.

The second synthetic target in this series was the ω-hydroxy analogue of compound **6**, which required a longer synthesis but is one of the compounds with better cellular activity. This compound could be envisioned as arising from homonerol through a sequence parallel to that used to obtain compound **15**. However, our previous route to homonerol employed an 8-step sequence[45] and while it gave isomerically pure material, to avoid an even longer sequence an alternative route to homonerol was developed. For this route, nerol (**16**) first was oxidized to neral (**17**) under either of two reaction conditions. A TEMPO oxidation[46] gave the desired aldehyde in just four hours while an MnO_2 oxidation required several days but gave the same aldehyde in nearly quantitative yield. Wittig olefination of neral produced the isomerically pure triene **18** in high yield. Regioselective hydroboration-oxidation of the terminal double bond in the triene **18** gave homonerol (**19**) in modest yield,[47] but the brevity of this reaction sequence made that acceptable.

Once homonerol (**19**) was in hand, multiple attempts to accomplish a SeO_2 oxidation went unrewarded. Therefore, the reaction sequence employed in Scheme 1 was reorganized so that the selenium dioxide oxidation could be pursued at a later stage. Treatment of homonerol (**19**) with methanesulfonyl chloride and subsequent reaction of the mesylate with sodium bromide gave homoneryl bromide (**20**) in good yield.[21] At this stage of the sequence, the selenium dioxide mediated allylic oxidation[48] was modestly successful, and furnished the desired alcohol **21** in ~25% yield. While this yield might still be improved, once the alcohol **21** was in hand replacement of bromide with azide was performed and the product immediately was carried to the next step. For this click reaction, the alkyne **23** was synthesized by a known procedure from tetraethyl vinyl bisphosphonate (**22**) through a two-step process.[21] The click reaction then was conducted under standard conditions to afford the ester **24**. McKenna hydrolysis of the phosphonate esters provided the desired salt **25**.

Once the two new triazoles **15** and **25** were available, they were tested for their ability to disrupt protein geranylgeranylation in cell assays and to inhibit GGDPS in enzyme assays. The activity of the new ω-hydroxy compounds was compared directly to their respective parent compounds **3** and **6**. As shown in Figure 2, the disruption of protein geranylgeranylation was evaluated using two methods: 1) immunoblot analysis for unmodified Rap1a (a representative substrate for geranylgeranyl transferase (GGTase) I; and, 2) ELISA for intracellular lambda light chain levels as a marker for disruption of Rab geranylgeranylation.[14] Lovastatin was included as a positive control.[14] Both compounds **15** and **25** induced concentration-dependent effects in these assays, consistent with GGDPS inhibitory activity. In both cases, the addition of the ω-OH group did modestly diminish cellular potency, with compound **15** approximately 10-fold less potent than **3** and compound **25** approximately 10-fold less potent than **6**. Enzyme assays utilizing recombinant GGDPS and FDPS confirmed the specificity of these new compounds as GGDPS inhibitors (Table 1).

Because it was more readily available, we then joined the alcohol **15** to HA (**26**, 10K) via NHS/EDC conjugation chemistry[49–52] to prepare the drug conjugate **27**. Conjugation was confirmed by the presence of the aromatic signal from the triazole moiety and acetyl

hydrogens from HA in the ^{1}H-NMR spectrum as well as by the presence of a phosphonate resonance in the ^{31}P NMR spectrum.

Next, we compared the cellular activity of the parent ω-OH compound **15** to the HA conjugate **27.** As shown in Figure 3, the HA-conjugate **27** induced greater accumulation of unmodified Rap1a than the free drug **15** in two different human myeloma cell lines. This enhanced cellular potency is likely a consequence of improved cellular uptake due to CD44 cell surface expression. Presumably cellular esterases[53–54] then hydrolyze the ω-OH-GGDPS inhibitor from the polymer, releasing free drug in the cell.

In conclusion, after introduction of the ω-hydroxyl group to geranyl acetate (**7**), and its immediate protection as a TBS ether, the synthetic sequence to compound **15** closely parallels our earlier synthesis of compound **3**. In contrast, the preparation of compound **25** employs a different synthetic sequence for preparation of homonerol (**19**), a sequence that is just three steps long rather than the eight used previously. As a result, this intermediate was available in sufficient quantity that the disappointing yield for the selenium dioxide oxidation to afford compound **21** could be tolerated. The remaining steps in the sequence then run parallel to our earlier synthesis of compound **6**.

While our prior studies have included substantial structure-function analysis of the triazole bisphosphonate class of GGDPS inhibitors, we have not previously evaluated the impact of modification of the terminal component of the isoprenoid chain. In the studies presented here we demonstrate that the addition of an ω-hydroxyl group to two of our previously reported inhibitors results in retention of GGDPS inhibitory activity. While the potency of these new analogues is diminished relative to the parent compounds, the hydroxy group affords the ability to conjugate these inhibitors to other agents such as HA with the goal of modifying drug biodistribution patterns and enhancing the therapeutic index. Our preliminary studies with the conjugate **27** demonstrate enhanced cellular activity of the HA-GGDPS inhibitor conjugate compared to the free drug. Future studies now can examine the biodistribution and toxicity profile of the HA-GGDPS inhibitor conjugates *in vivo*, as well as explore alternative modifications at the ω-position to create additional “linkable” inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Jack Neal for his assistance with preparation of some of the synthetic intermediates. This project was supported in part by the Roy J. Carver Charitable Trust (01-224 to DFW) and the National Institutes of Health (R01CA-172070 to SAH, P20 GM103480 and P30 CA036727). DB was supported through a University of Nebraska Medical Center Program of Excellence Assistantship, and WMP was supported by fellowships from the PhRMA Foundation and Blue Waters Foundation.

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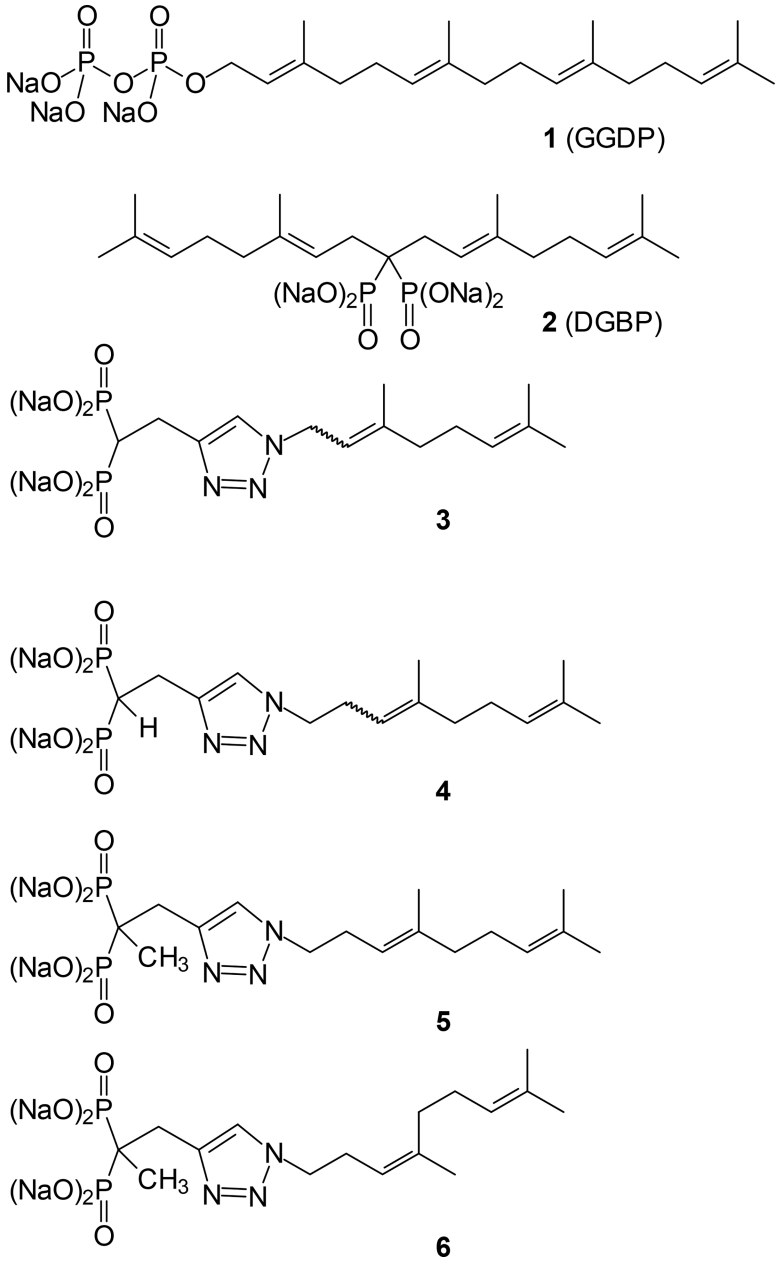


Figure 1.
Geranylgeranyl diphosphate and known inhibitors of geranylgeranyl diphosphate synthase.

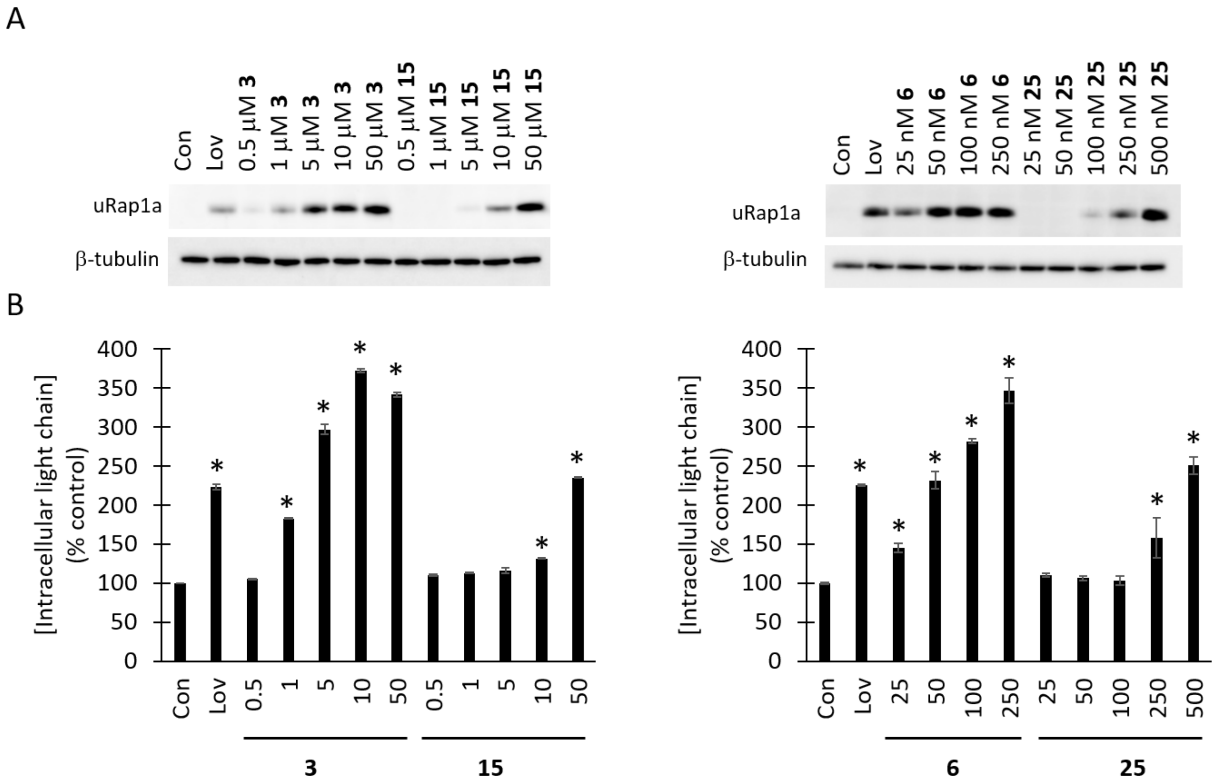


Figure 2. Comparison of the effects of the novel ω-hydroxyl triazole bisphosphonates to the parent analogues on protein geranylgeranylation.

RPMI-8226 cells were incubated for 48 hours in the presence or absence of lovastatin (*Lov,* 10 μM) or varying concentrations of the test compounds. A) Immunoblot analysis of uRap1a (antibody detects only unmodified protein) and β-tubulin (as a loading control). B) Intracellular lambda light chain concentrations were determined via ELISA. Data are expressed as a percentage of control (mean ± SD, n=3). The * denotes $p < 0.05$ per unpaired two-tailed *t*-test.

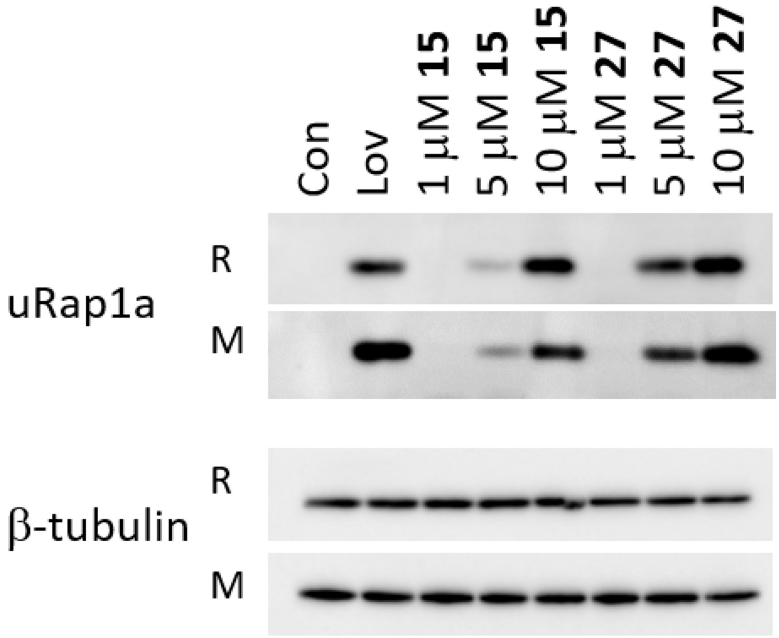
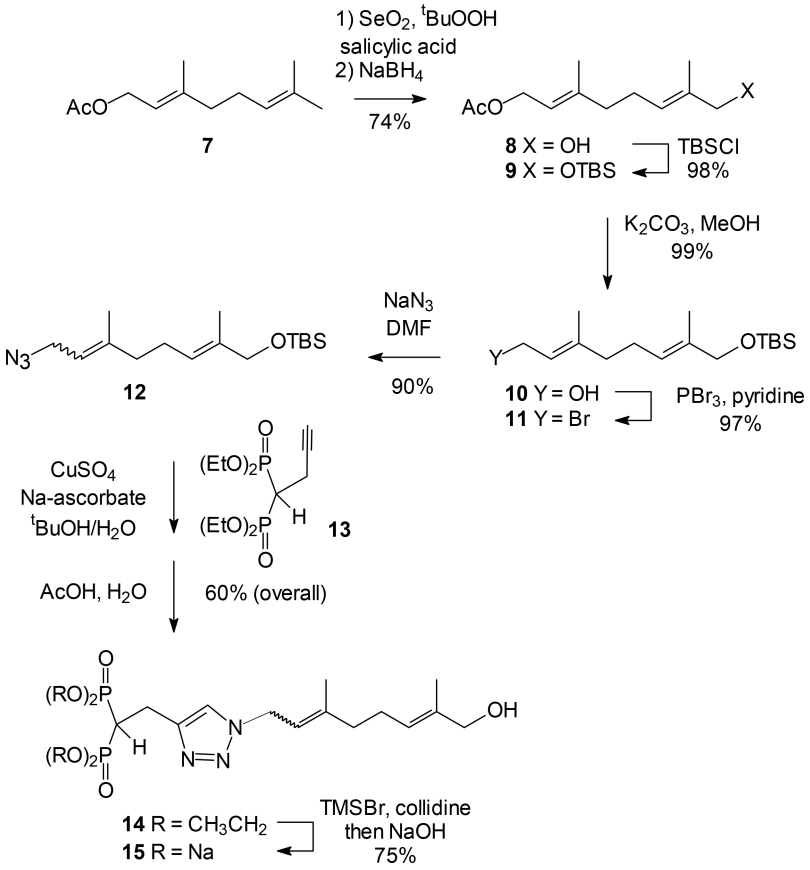


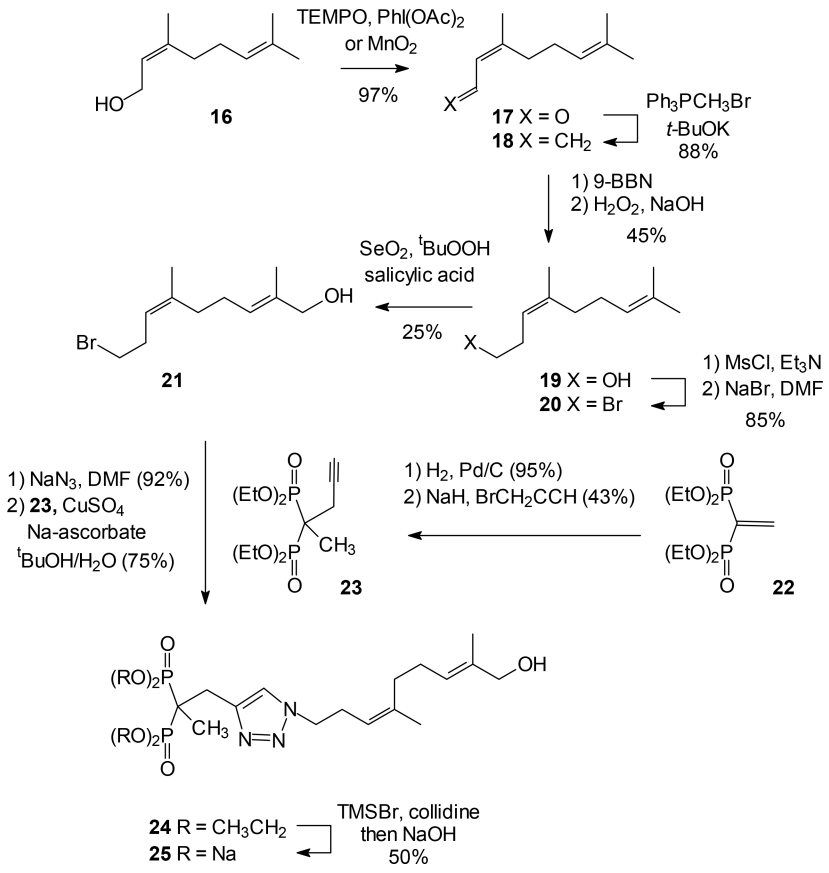
Figure 3. Comparison of the effects of the ω-hydroxyl triazole bisphosphonate 15 and the HA-conjugate 27 on protein geranylgeranylation.

RPMI-8226 (R) or MM.1S (M) cells were incubated for 48 hours in the presence or absence of lovastatin (*Lov,* 10 μM) or varying concentrations of the test compounds (for the HA-conjugate **27**, concentration is based on the percent weight of the inhibitor in the HA polymer conjugate). Immunoblot analysis of uRap1a (antibody detects only unmodified protein) and β-tubulin (as a loading control). These blots are representative of three independent experiments.



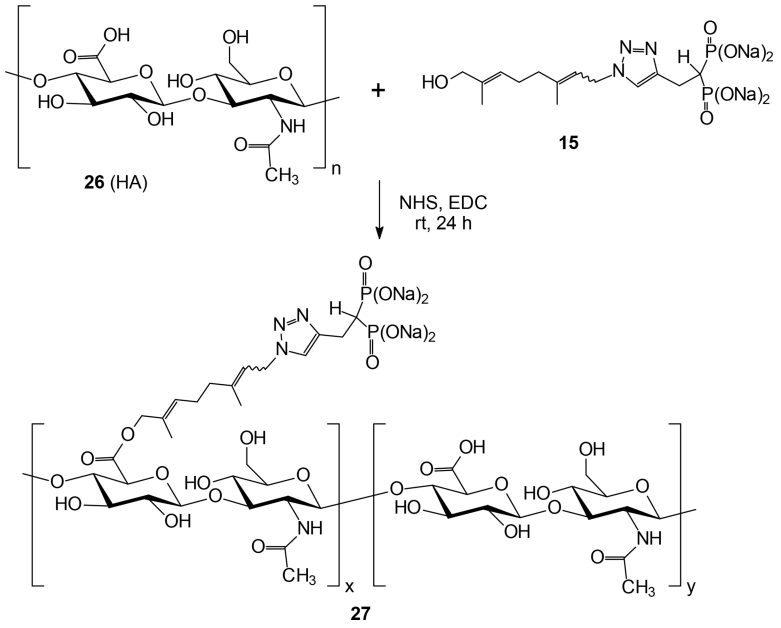
Scheme 1.

Synthesis of compound **15**, the ω-hydroxy analogue of compound **3.**



Scheme 2.

Synthesis of compound **25**, the ω-hydroxy analogue of compound **6.**



Scheme 3.

Generation of HA-GGDPS inhibitor polymer conjugate **27**.

Table 1.

Summary of the bioassay results of the novel ω-OH-triazole bisphosphonates.

Compound	GGDPS IC^{50} (μM)	FDPS IC^{50} (μM)	Fold-selectivity for GGDPS compared to FDPS	Cellular LEC^{1} (μM)
15	11.6 + 2.6	91.2 + 25.0	8	10
25	0.67 + 0.30	81.4 + 23.5	121	0.25

[1]Cellular LEC (lowest effective concentration) 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.