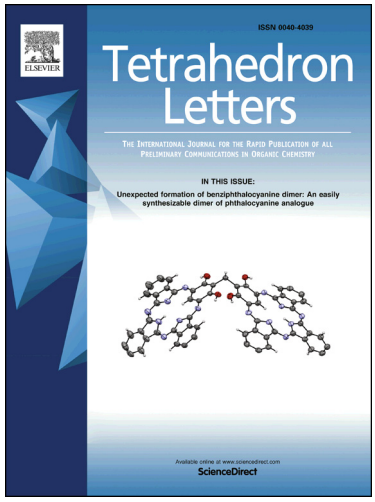

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Graphical Abstract

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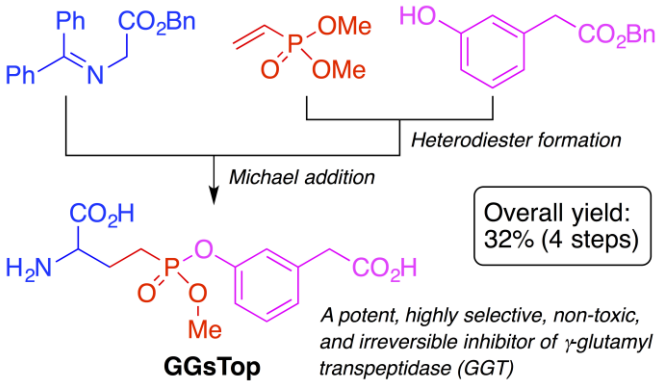

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An improved synthesis of the potent and selective γ-glutamyl transpeptidase inhibitor GGsTop together with an inhibitory activity evaluation of its potential hydrolysis products

Bunta Watanabe[a, 1]*, Tatsuya Morikita[a], Yukiko Tabuchi[a], Ryoto Kobayashi[b], Chunjie Li[a], Masakazu Yamamoto[b], Takao Koeduka[a, 1] and Jun Hiratake[a]

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ABSTRACT

A previously reported synthetic route to 2-amino-4-{[3-(carboxymethyl)phenoxy](methoxy)phosphoryl}butanoic acid (GGsTop), a potent, highly selective, non-toxic, and irreversible inhibitor of γ-glutamyl transpeptidase (GGT) was substantially improved. This route furnishes GGsTop in four steps with an overall yield of 32% from inexpensive starting materials, i.e., the yield is increased approximately sixfold relative to the previous protocol. The synthesis and inhibitory activity evaluation of potential hydrolysis products of GGsTop clearly demonstrated that GGsTop is the active inhibitor, and the conceivable hydrolysis products barely affect the activity of human GGT.

Introduction

2-Amino-4-{[3-(carboxymethyl)phenoxy](methoxy)phosphoryl}butanoic acid (GGsTop; Scheme 1)[1, 2] is a potent, highly selective, non-toxic, and irreversible inhibitor of γ-glutamyl transpeptidase (GGT; EC 2.3.2.2),[3-6] which is a key enzyme in glutathione homeostasis. Since glutathione is a fundamental tripeptide that mediates not only the redox balance of cells, but also the detoxification of xenobiotics and active oxygen species, abnormally high levels of GGT may damage cells by depletion of glutathione and elevated oxidative stress. It is therefore not surprising that GGT dysfunction has been correlated to many physiological disorders such as cancer,[3, 6-9] diabetes,[3] hepatitis,[3] nephritis,[3] as well as respiratory[10] and cardiovascular diseases.[3, 11, 12] Therefore, GGT represents an attractive pharmaceutical target, while GGsTop, a mechanism-based non-toxic inhibitor of GGT, is a promising prospective chemotherapeutic agent for a vast variety of diseases related to GGT activation.

The synthesis of GGsTop has previously been described in a patent,[13] but even though GGsTop is now commercially available, its synthesis remains relatively laborious and inefficient. Nevertheless, GGsTop has been used in studies focusing on GGT and GGT-related disorders. For example, GGsTop has been successfully used for the determination of the crystal structure of GGsTop-bound human GGT.[14] Furthermore, we have previously demonstrated that GGsTop exhibits protective effects against ischemia/reperfusion-induced renal and hepatic injury in rat models.[15, 16] We have also reported that GGsTop attenuates air way hyper-reactivity and air way epithelial cell mucous accumulation in a mouse allergic asthma model.[17] In these cases, the administration of GGsTop suppressed the GGT activation and increased the glutathione content.

GGsTop also exhibits beneficial effects on human skin cells.[18] For example, a treatment of human skin fibroblasts with GGsTop induces the synthesis of type I collagen and elastin, and it increases the expression of α-smooth muscle actin and heat-shock protein 47, a collagen-specific molecular chaperone. Furthermore, GGsTop enhances the migration of human epidermal keratinocytes. The effects of GGsTop on human skin cells render it an attractive agent for the treatment of aging skin, and GGsTop is thus used as an active ingredient (Nahlsgen) in anti-aging cosmetic products that are currently marketed in Japan.[19]

In the case of human skin fibroblasts, a GGsTop treatment temporarily decreases the glutathione content and elevates the concentration of reactive oxygen species. This slight oxidative stress has been proposed to elicit an anti-oxidative pathway, and to induce cell responses such as the biosynthesis of collagen.[18] Similar cell-activating effects of GGsTop on human periodontal

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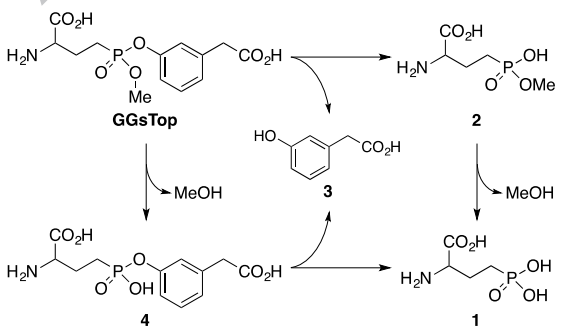
ligament cells have recently been reported: GGsTop treatment increases their migration and enhances the expression levels of type I collagen and α-smooth muscle actin, both of which play vital roles in wound healing.[20] The effects of a treatment with GGsTop seem to depend on the experimental conditions including the tissue/cell type. Nevertheless, a GGsTop treatment ultimately generates protective effects against oxidative stress, and it is worth mentioning that no evidence for toxicity was observed in any of the aforementioned cases. Therefore, GGsTop should find a multitude of implementations in both fundamental research and practical applications in the near future.

Although we have previously reported two independent synthetic routes to GGsTop,[1, 13] both are relatively laborious and/or inefficient. The first route furnishes GGsTop in five steps with an overall yield of 11% starting from 2-amino-4-(dihydroxyphosphryl)butanoic acid (**1**; Scheme 1),[1] whereby **1** has to be prepared from diethyl 2-bromoethylphosphonate in three steps with an overall yield of 47%.[21] This reported method thus requires a total of eight steps and affords GGsTop in a combined maximum yield of 5.2%. The bottleneck of this reaction protocol is the formation of a phosphonic acid heterodiester from a phosphonic acid dichloride, which proceeds in merely 31%.[1] While this synthesis is academically interesting, it is of low utility in an industrial context, given its low yield, high number of steps, and the fact that the cost of commercially available **1** is prohibitively high to prepare GGsTop on a multigram scale.

We therefore developed a shorter synthesis for the commercial production of GGsTop. This second route affords GGsTop from dimethyl vinylphosphonate, and generates the glutamate mimic moiety of GGsTop via a Michael addition of a glycine imine species to a vinylphosphonate derivative.[13] This alternative synthetic route is already less laborious than the first (from 8 to 4 steps), and despite the low yield (18%), it is commercially viable, given the low cost of the starting materials. Nevertheless, considering the benefits of GGsTop and its anticipated applications, an improved and more efficient synthesis of GGsTop should be highly desirable in order to reduce production costs.

Furthermore, it remains to be determined whether potentially formed hydrolysis products of GGsTop also inhibit the activity of human GGT. The probable hydrolysis of GGsTop is outlined in Scheme 1. On one hand, hydrolysis of the phosphonic aryl ester could afford mono-methyl ester **2** under concomitant release of 3-hydroxyphenylacetic acid (**3**). On the other hand, hydrolysis of the phosphonic methyl ester could generate phosphonic mono-aryl ester **4**. Both **2** and **4** should afford **1** upon further hydrolyzation. So far, the inhibitory activity of **1**–**4** against human GGT has not been investigated.



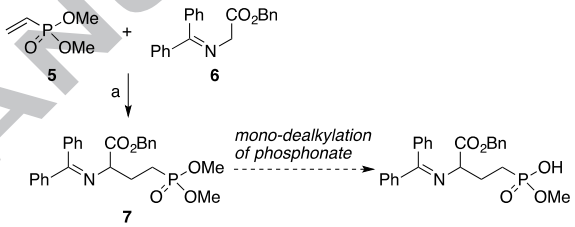
Scheme 1. The structure of GGsTop and potential hydrolysis products **1**-**4**.

In this study, we developed a synthetic route to GGsTop from inexpensive starting materials, which is significantly more efficient and/or less laborious than previously reported protocols.[1, 13] We also synthesized **2** and **4**, and evaluated the inhibitory activity of the potential hydrolysis products **1**–**4** against human GGT.

Results and discussion

The first part of our new synthetic protocol is outlined in Scheme 2. Similar to our previous method,[13] commercially available and moisture-stable dimethyl vinylphosphonate (**5**), which can be easily handled, was selected as the source of the phosphonate moiety in GGsTop. In order to overcome the low yield associated with the formation of the phosphonic acid heterodiester in the previously reported GGsTop synthesis,[1] we attempted to use a mono-dealkylation of the phosphonic acid diester to the corresponding monoester as a key reaction. Prior to the mono-dealkylation trials, the construction of the 2-amino-4-phosphonobutanoic acid moiety of GGsTop via a Michael addition of glycine imine **6** to vinylphosphonate **5** was examined as the other key reaction (Scheme 2).

Only a few examples concerning the Michael addition of



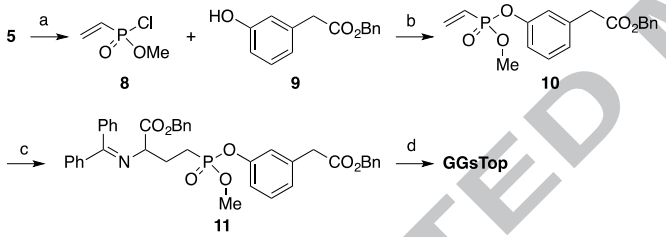
Scheme 2. The first part of our new synthetic route; reagents and conditions: (a) $[BnEt_3N]Cl$, K_2CO_3 (3 eq), MeCN, reflux, 4 h, 83%.

glycine imines to vinylphosphonates or vinylphosphinates have been reported so far. Sturtz and Guillamot have reported the Michael addition of a benzaldehyde imine of glycine ethyl ester to diethyl vinylphosphonate in the presence of ethoxide anions in ethanolic solution.[22] The conjugate addition of benzophenone imine of glycine ethyl ester to methyl methylvinylphosphinate was reported to proceed in ethanol using potassium hydroxide as a base.[23] Hamilton *et al*. have reported the Michael addition of glycine benzophenonimine derivatives to vinylphosphonates using potassium hexamethyldisilazide at –78 ºC, which proceeded in excellent yields.[24] In the present study, the basic conditions in alcoholic media were discarded in order to prevent ester exchange reactions. In addition, we aimed to avoid the use of strong bases and relatively low reaction temperatures, e.g. –78 ºC, in order to develop an economical and operatively easy process. With these conditions in mind, we applied the phase-transfer protocol reported by Jiang and co-workers,[25] although they did not examine vinylphosphonates as Michael acceptors. As shown in Scheme 2, the Michael addition of benzophenone imine **6** to vinylphosphonate **5** proceeded smoothly in boiling acetonitrile in the presence of benzyltriethylammonium chloride as the phase-transfer catalyst and three equivalents of potassium carbonate as the base. Michael adduct **7** was obtained in 83% yield after chromatographic purification.

Then, we examined the mono-dealkylation of phosphonic acid dimethyl ester **7** in the presence of benzyl carboxylate. As sodium iodide is a typical reagent for such transformations,[26, 27] we treated **7** with one equivalent of sodium iodide in refluxing 2-butanone. Unfortunately, the precipitate formed during the reaction was relatively unstable, and rapidly decomposed during the attempted isolation. We also tested *tert*-butylamine[28-30]

instead of sodium iodide, but little conversion was observed, even after 5 days. We further subjected dialkyl phosphonate **7** to a direct monochlorination, in the hope to use the resulting chloride directly for the next heterodiester formation step. However, treatment of **7** with oxalyl chloride[31-33] or dichlorotriphenylphosphorane[34] leads to the decomposition of **7** and the desired product was not obtained. These results indicate that the acid-sensitive diphenylmethylene group of **7** is unstable under these conditions. At this point, we decided to change the initial synthetic plan, and to generate the phosphonic acid heterodiester prior to the Michael addition.

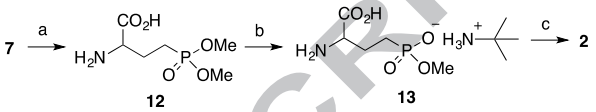
This revised plan, the improved version of our previous route,[13] is illustrated in Scheme 3. In this case, the monochlorination of the dialkyl phosphate instead of mono-dealkylation of phosphonic acid diester was selected as a key step in order to improve the yield of the formation of the phosphonic acid heterodiester. The other key reaction is the Michael addition of glycine imine **6** to the heterodiester of vinylphosphonic acid. In the first key step, dimethyl vinylphosphonate (**5**) was converted into methyl vinylphosphonochloridate (**8**)[13, 30, 35] by treatment with oxalyl chloride.[31-33] Replacement of dichloromethane with 1,2-dichloroethane as the solvent shortened the reaction period from 3 days[13] to 30 h without affecting the yield of **8**. Subsequently, **8** was treated with benzyl 3-hydroxyphenylacetate (**9**). The decreased reaction time (16 h to 2 h), which probably prevents the decompsion of the product, improved the yield of heterodiester **10** from 87%[13] to almost quantitative.



Scheme 3. Improved synthesis of GGsTop; reagents and conditions: (a) $(COCl)_2$, $ClCH_2CH_2Cl$, 60 ºC, 30 h, 53%; (b) Et_3N, CH_2Cl_2, 16 ºC, 2 h, 99%; (c) **6**, $[BnEt_3N]Cl$, K_2CO_3 (2 eq), MeCN, 20–26 ºC, 6.5 h, 67%; (d) H_2, Pd-C, MeOH, rt, 4 h, 90%.

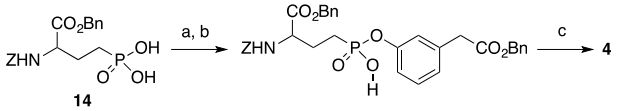
The second key step of the synthesis is the Michael addition of glycine imine **6** to **10**. Under the aforementioned catalytic phase-transfer conditions (*vide supra*), heterodiester **11** was obtained in 42% yield after chromatographic purification.[13] An extensive examination of the reaction conditions revealed that the reaction proceeds at 20–30 ºC with a satisfactory rate in the presence of a reduced amount of potassium carbonate (3 to 2 eq). Addition of **10** into a pre-prepared mixture of the other reactants also proved to effective, and the yield of **11** was thus improved to 67%. The results indicate that higher reaction temperatures and an excess of base lead to the decomposition of the relatively reactive arylphosphonates **10** and **11**. Finally, all protecting groups in **11** were removed under hydrogenolysis conditions to furnish GGsTop in 90% yield after purification by reverse-phase chromatography. In consequence, we have considerably improved the previously reported synthetic routes to GGsTop,[1, 13] i.e., the overall yield of GGsTop in this four-step synthetic route is ensured to be at least 32%. A further optimization of each reaction step should increase the total yield even further. It has been reported that asymmetric Michael additions of glycine imine derivatives such as **6** to various acrylates using chiral catalysts proceed with high stereoselectivity.[36, 37] Applying these conditions, it should be possible to introduce chirality at the α-carbon atom of the α-amino acid moiety of GGsTop.

In the synthesis of **2**, previously prepared **7** was used as a synthetic precursor (Scheme 4). The benzylic protecting groups of **7** were removed by hydrogenolysis to furnish **12**[29] in 68% yield. The mono-dealkylation of the phosphonic acid dimethyl ester moiety was achieved by treating **12** with boiling *tert*-butylamine[28-30] to afford ammonium salt **13**, which was converted into **2**[29] by ion-exchange column chromatography. In this study, **2**, **12**, and **13** were obtained as crystalline solids, even though **2** and **12** have previously been prepared by independent synthetic routes and been reported as viscous oils.[29]



Scheme 4. Synthesis of **2**; reagents and conditions: (a) H_2, Pd-C, MeOH/H_2O, rt, 3 h, 68%; (b) *t*-$BuNH_2$, MeOH, reflux, 2 days, 79%; (c) Dowex 50Wx8 (H^+ form), H_2O, 94%.

The synthesis of **4** is illustrated in Scheme 5. **14**, the key intermediate of our previously reported GGsTop synthesis,[1] was treated with oxalyl chloride to give a phosphonic acid dichloride, which was successively treated with one equivalent of phenol **9** and excess water to give the corresponding mono-ester intermediate. The ensuing removal of the protecting groups afforded **4** in 46% yield from **14** after chromatographic purification.



Scheme 5. Synthesis of **4**; reagents and conditions: (a) $(COCl)_2$, DMF, CH_2Cl_2, rt, 3 h; (b) i. **9**, Et_3N, CH_2Cl_2, –78 ºC to rt, 1 h; ii. H_2O, rt, 2 h; (c) H_2, Pd-C, MeOH/H_2O, rt, 3 h, 46% from **14**.

The inhibitory activity of **1**–**4** against human GGT was measured at pH = 5.5 according to a previously described method.[1, 38, 39] As GGsTop is a slow-binding inhibitor, the second-order rate constant for enzyme inactivation (k_{on}) was used as an index of inhibitory activity. Among the possible hydrolytic products of GGsTop, only mono-aryl ester **4** exhibited a weak inhibitory activity ($k_{on} = 0.58 \pm 0.03\ M^{-1}s^{-1}$), while **1**–**3** were inactive, even at concentrations as high as 0.1 mM. Compound **4** is by a factor of 88 less potent than GGsTop ($k_{on} = 51 \pm 3\ M^{-1}s^{-1}$),[1] even though **4** bears a 3-hydroxyphenylacetic acid, which is an ideal mimic of the cysteinylglycine of glutathione. We have recently reported that the negative charge on the carboxy group of the 3-hydroxyphenylacetic acid moiety allows GGsTop to interact with the positive charge of Lys562 at the active site of GGT, and that this electrostatic interaction plays a critical role in GGsTop recognition by human GGT.[2] The great decrease of the inhibitory activity of **4** should hence be due to the presence of the negative charge on the oxygen atom of the phosphonate moiety under the assay condition (pH = 5.5), considering that the pK_1 values of simple phosphonic acids are 2.3-2.9.[40] As previously mentioned,[1] the negative charge should decrease the affinity of **4** for the human GGT active site by electrostatic repulsion, and/or by disfavoring the nucleophilic attack of the hydroxy group of Thr381, which is the active site nucleophile of human GGT,[41] on the phosphorus atom of **4** in the GGT deactivation process. These results clearly demonstrate that GGsTop itself is the active inhibitor, and that potential hydrolytic products barely affect human GGT.

Conclusion

In summary, the route established in this study uses inexpensive starting materials and furnishes GGsTop in four steps with an overall yield of 32%. We also synthesized potential hydrolysis products of GGsTop, and demonstrated that they possess very limited inhibitory activity against human GGT. Currently, GGsTop is synthesized on a kilogram scale by the route developed in this study, and used as an active ingredient (Nahlsgen) in anti-aging cosmetic products that are marketed in Japan.

Acknowledgments

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Highlights

Improvement of a reported synthesis of γ-glutamyl transpeptidase inhibitor GGsTop.

The improved route furnishes GGsTop in four steps with an overall yield of 32%.

The key reaction step: a Michael addition under catalytic phase-transfer conditions.

The Michael acceptor: an alkyl(aryl)heterodiester of vinylphosphonic acid.

Conceivable hydrolysis products of GGsTop barely affect the activity of human GGT.