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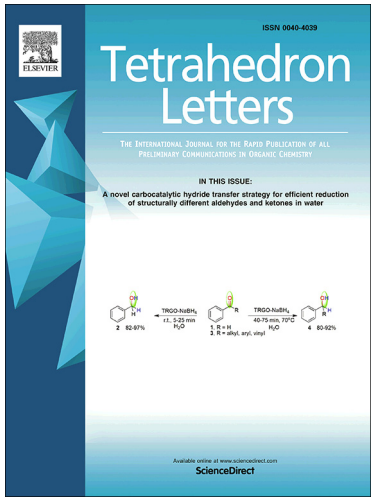

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Synthesis of Gentianine *N*-Oxide by Enzymatic Hydrolysis of Swertiamarin in the Presence of Hydroxylamine and Reaction Pathway to Gentianine and Gentianol

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ABSTRACT

Gentianine is a metabolite of gentiopicroside and swertiamarin. Several biological activities have been reported for gentianine, such as antiinflammatory and antidiabetic activity, and hypotensive effect. Gentiopicroside is found in 0.9–9.8% content in Gentian root or Gentian scabra root, and Swertiamarin is contained in Swertia herb in 2–10%. These natural products can be potential starting materials for the synthesis of gentianine. This study describes the β-glucosidase-catalyzed hydrolysis of gentiopicroside and swertiamarin in the presence of hydroxylamine to afford gentianine *N*-oxide, which can be a synthetic precursor of gentianine derivatives. Enzymatic hydrolysis of swertiamarin selectively afforded gentianine *N*-oxide in 81% yield, whereas gentiopicroside afforded gentianine *N*-oxide and gentianol *N*-oxide. Plausible reaction pathways leading to gentianine, gentianol, and their *N*-oxides were also investigated.

1. Introduction

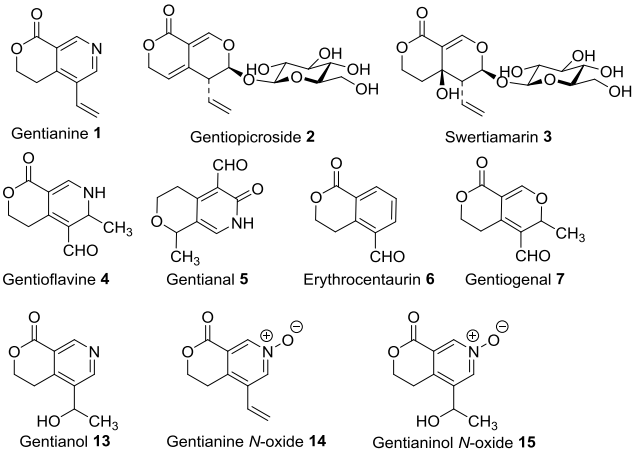
Gentianine (**1**) is a metabolite of gentiopicroside (**2**) and swertiamarin (**3**), which are secoilidoid glycosides known as constituents of Gentianaceae plants (Fig. 1).[1-5] For **1**, several biological activities have been reported, such as antiinflammatory[6,7] and antidiabetic activity,[8] hypotensive effect,[9] and protective effect on hippocampal CA1 neurons in rats.[10] The antidiabetic activity of **1** stems from the fact that it increases adipogenesis, which has been associated with a significant increase in the mRNA expression of PPAR-γ, GLUT-4, and adiponectin. For further medicinal studies, the synthesis of derivatives of **1** is desired.

The synthesis of **1** from 4-methyl-5-vinylnicotinonitrile has been previously reported.[11,12] However, several steps are generally required. On the other hand, **1** was synthesized by ammonolytic cleavage of above-mentioned glycosylated form (**2** and **3**) in low yield due to the formation of gentioflavine (**4**) and gentianal (**5**) as byproducts (Fig. 1).[13,14,15] Gentiopicroside **2** is found in 0.9–9.8% content in Gentian root or Gentian scabra root,[16,17] and swertiamarin **3** is contained in Swertia herb in 2–10%.[5] These natural products are potential starting materials for the synthesis of **1** and its derivatives.

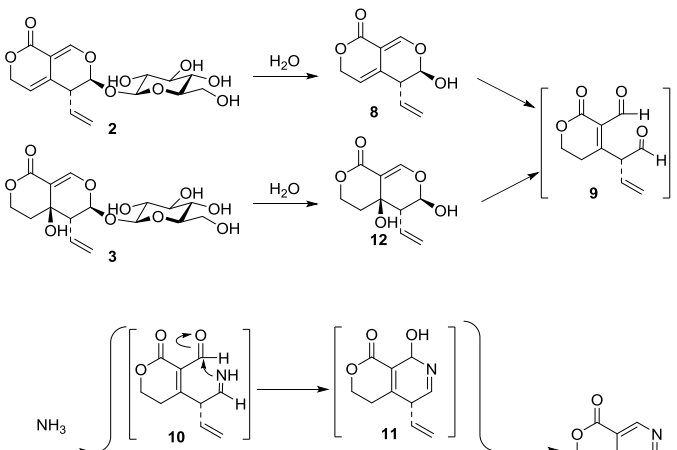
In the absence of ammonia, the formation of **1** is a result of sequential reactions initially triggered by the hydrolysis of **2** or **3** that affords many kinds of byproducts such as erythrocentaurin (**6**)[4,18] and gentiogenal (**7**)[19] (Fig. 1). Therefore, enzymatic hydrolysis of **2** or **3** in ammonium buffer can be envisaged as a very interesting and challenging approach (Scheme 1); the use of a degradative and harmless natural product and the mild reaction conditions of an

Fig. 1. Structure of gentianine **1**, gentiopicroside **2**, sweritamarin **3**, and the derivatives.

enzymatic reaction are preferred from the viewpoint of green chemistry.[20] Several biotransformations or biodegradations of **2** and **3** by intestinal bacteria have been reported,[2-5] and the mechanism involved in the production of **1** from **2** or **3** is depicted in Scheme 1. Accordingly, the hydrolysis of **2** by β-glycosidase gives aglycon **8**, which undergoes hemiacetal bond



cleavage to yield dialdehyde **9,** whose condensation with ammonia forms imines **10** and/or **10'**. Then, intermolecular cycloaddition affords dihydropyridines **11** and/or **11'**, which provide **1** after aromatization. In a similar manner, the hydrolysis of **3** gives **1** through **12**. Although gentianol **13** is also known to be produced by biodegradation, the pathway leading to its formation is unclear.[5]



Scheme 1 Proposed mechanism for the enzymatic hydrolysis of **2** and **3**.

In our preliminary experiments regarding the enzymatic hydrolysis of **2** or **3**,[21] two problems arose: the low yield of **1** and inhibition of **2** to enzyme. Hydrolytic intermediate **9** may react with the lysine residue of the enzyme, since it has been known to produce an adduct with ethylamine. In this paper, we investigated the reaction pathway affording **1** and **13** by enzymatic hydrolysis of **2** and **3** in the presence of ammonia and moreover the formation of pyridine *N*-oxide derivatives **14** and **15** (Fig. 1) in the presence of hydroxylamine expecting to facilitate the subsequent reactions.

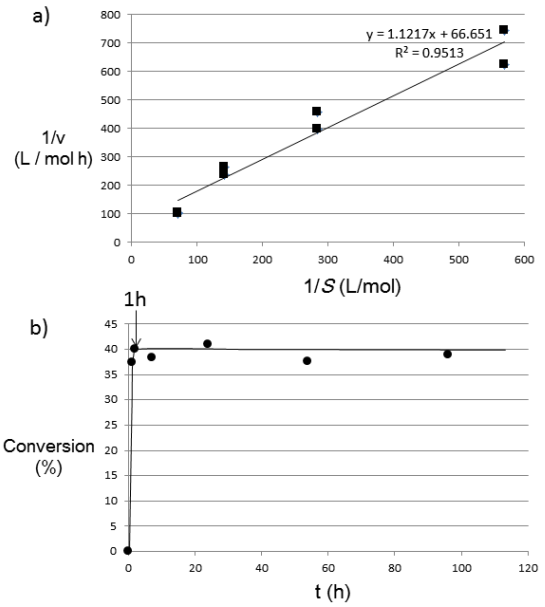
2. Results and discussions

2.1. Kinetics and time course of the enzymatic hydrolysis of 2

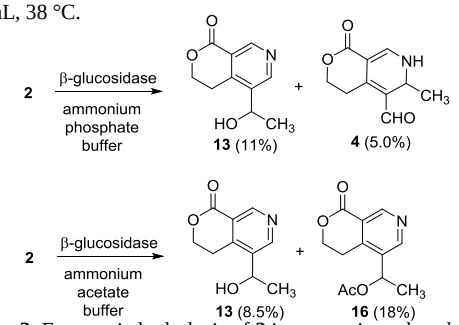
Firstly, to investigate the reactivity of **2** and inhibition of the products to glucosidase, the kinetics and time course of the enzymatic hydrolysis of **2** using almond β-glucosidase purchased from Oriental yeast Co. Ltd. (Product No. 46361003, lot. 3459401, 36.8 U/mg) were studied. Initial rate kinetics were performed. The ratio of consumed **2** was measured by ^{1}H NMR spectroscopy using propane-1,2-diol as an internal standard. The remaining amount of **2** (or **3**) was calculated by using the integral of the signal at 7.6 ppm (singlet, 3-position). The K_m and V_{max} values in the conditions of the experiment were determined to be 17 mmol/L and 15 mmol/L·h, respectively (Fig. 2a). The time course of the reaction, which was monitored starting from 14 mmol/L of **2**, shows that the reaction stops within one hour (Fig. 2b), which is most likely due to product inhibition because the enzyme could be stored in the same buffer for one hour without significant loss of activity. Although β-glucosidase exhibited enough ability to hydrolyze **2**, successive addition of the enzyme and the use of a more reactive nitrogen reagent seemed necessary to enhance the enzymatic hydrolysis of **2** and **3**. Unfortunately, measurement of the kinetics of **3** was hindered by a stronger inhibition by hydrolyzed products and slower reaction rate of **3** compared with that of **2**.

2.2. Enzymatic hydrolysis of 2 and 3 in the presence of hydroxylamine

To improve the efficiency of pyridine ring formation, ammonia was replaced with hydroxylamine due to the higher nucleophilicity of the latter compound. To the best of our knowledge, a hydrolysis reaction catalyzed by β-glycosidase in the presence of hydroxylamine has not been reported so far. The results of the enzymatic hydrolysis are listed in Table 1. Entries 1 and 2 in Table 1 shows the yield and conversion of products and the recovery of the substrate in the hydrolysis of **2** and **3**, respectively, at pH 5.5. The conversion to **14** was calculated on the basis of the consumed substrate (added **2** or **3** minus recovered **2** or **3**). Although hydrolysis of **2** afforded gentianine *N*-oxide (**14**) and gentianol *N*-oxide (**15**)[22] in 32% and 38% yields, respectively, that of **3** gave **14** as the sole product in 52% yield. Compound **14** was previously reported as a degradation product of **1**, and the spectral data of **14** was identical to those found in the literature.[2] Swertiamarin **3** proved to be a much better substrate than gentiopicroside **2** for the synthesis of **14**. Next, the effect of pH (entry 2–5, Table 1) and the amount of hydroxylamine (entry 6–10, Table 1) on the yield and conversion in the hydrolysis of **3** were investigated. We found that one equivalent of hydroxylamine reacted with glucose and excessive addition of hydroxylamine (entry 11, Table 1) resulted in the occurrence of side reactions with the concomitant decrease of the yield. It can be extracted from the table that the reaction at pH 6 and the use of 2.4 equivalents of hydroxylamine (entry 3, Table 1) provided the best results (71% yield and 82% conversion). By adding fresh enzyme every 30 min for 3 h, the isolated yield reached 81%. The high conversion was achieved because both oxime formation and β-glucosidase-catalyzed hydrolysis are the preferred processes in the range of pH 4–6. Generally, pyridine *N*-oxides are reactive precursors of pyridine derivatives through, for example, acetyl migration and aromatic electrophilic substitution.



a) Linweaver–Burk plot of initial rate kinetics. Conditions: **2**; 1.8–14 mmol/L, almond β-glucosidase from Oriental yeast Co. Ltd., 39 U/mg; 55 U/L, 0.2 M ammonium phosphate buffer (pH 5.5); 10 mL, for 30 min at 38 °C (consumed **2**; 10%–35%). b) Time course. Conditions: **2**; 14 mmol/L, almond β-glucosidase; 110 U/L, 0.2 M ammonium phosphate buffer (pH 5.5); 10 mL, 38 °C.



Scheme 2. Enzymatic hydrolysis of **2** in ammonium phosphate or acetate buffer.

2.3. Reaction pathway toward 1, 13, 14, and 15.

Despite being a known metabolite from **2** and **3**, the degradation pathway of gentianol **13** has not been well investigated. To understand the selectivity of the hydrolysis reaction in the presence of hydroxylamine to afford **14** and **15**, the elucidation of the mechanism of the enzymatic hydrolysis giving **13** is important.

To this aim, the enzymatic hydrolysis of **2** in the presence of ammonium acetate was tested. Compound **2** (100 mg) in ammonium acetate buffer (pH 5.5, 0.2 M, 20 mL) was incubated at 78 rpm and 38 °C, and enzyme solution (0.5 mg/100 μL buffer, 10 times) was added every 30 min until the substrate was consumed according to TLC analysis. Although **1** could not be

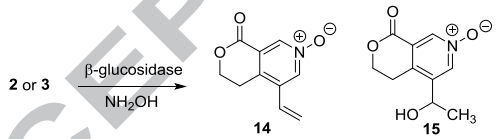
isolated, gentianol **13** and gentianol acetate **16**[23], were obtained in 8.5% and 18%, respectively (Scheme 2). Since the reaction in ammonium phosphate buffer affords **13**[5] (11%), **4**[14] (5%), and **6**[4] (1%), but not **16**, it can be concluded that the acetate group of **16** stems from the acetate buffer. Compounds **1, 13**, and **16** were proved to be noninterchangeable when treated individually under the same conditions of the enzymatic hydrolysis, which suggests that the pathways affording **1**, **13**, and **16** exist independently.

Scheme 3 depicts a plausible pathway to give **13** from **2**. In this pathway, dialdehyde **9**, which is derived from aglycon **8**, contains an acidic proton at the 9-position that could be activated by an electron withdrawing group such as an aldehyde and a conjugated ester or aldehyde. Thus, once the anion from **9** is formed, conjugated allyl addition to the aldehyde would afford erythrocentaurin (**6**)[4] or, alternatively, its isomerization would afford **17**, which could be a precursor of gentiogenal (**7**).[18,19] Since the vinyl group is normally inactive at pH 5.5 and room temperature, **7** would be most likely obtained through the intramolecular addition reaction of the activated olefin in **17** with the carbonyl group. During the ^{1}H NMR monitoring of the reaction (Fig. 2b), an intermediate possessing a doublet methyl signal at 1.35 ppm was observed at the beginning of the reaction, but it disappeared gradually and was no longer observed after three hours. Since this intermediate was stable enough to be detected in the NMR experiment, it is not likely to be the unstable species **17**. On the other hand, **13** was not observed at 30 min reaction time, but it appeared and its concentration increased while the intermediate disappeared, reaching a maximum conversion of ~3% after four hours. This result supports the isomerization of the vinyl group in 9 to the conjugated olefin in **17**. According to the pathway in Scheme 3, **17** would react with ammonia giving imine **18** or **18'**, which would be then converted into **19** and **19'**, respectively.[5,15] Aromatization by elimination of the hydroxyl group, followed by nucleophilic attack of water or acetate would afford **13** or **16**, respectively. Indeed, the specific rotation of **13** obtained from **2** was $[\alpha]_D$ = -0.7° (c = 0.4, $CHCl_3$){lit.[5] $[\alpha]_D$ = -64° (c = 1, $CHCl_3$), synthetic sample prepared by asymmetric reduction of the corresponding ketone.}, which supported the pathway through nonchiral intermediate **17**. Meanwhile, the enzymatic hydrolysis of **3** would afford **20**, which possesses weaker acidity at the 9-position than **9** due to the lack of conjugation with the ester and the aldehyde in this structure. Nevertheless, **20** would be dehydrated to afford **9**, which would in turn lead to **13**. On the other hand, the reaction of **20** with ammonia would afford **1** through intermediates **21** and **22**. Accordingly, the enzymatic hydrolysis of **3** afforded **1** and **13** in 8% and 12% yield, respectively.

Similar reaction pathways are proposed for the hydrolysis of **2** and **3** in the presence of hydroxylamine (Scheme 4). After the formation of dialdehyde **9** from **2**, two possible routes can be considered: isomerization to **17** or reaction with the highly reactive hydroxylamine to afford **14**. Considering the yield of the reaction (Table 1, entry 1), the isomerization is more favored than the reaction with hydroxylamine. On the other hand, in the reaction of **3**, intermediate **20** would react exclusively with hydroxylamine to afford **14** due to its lower acidity in comparison with **9**.

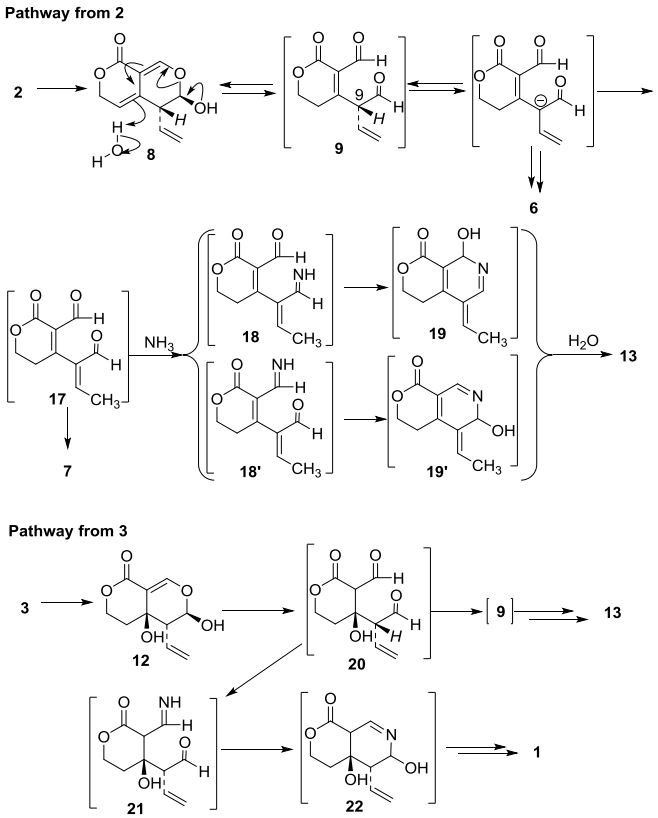
It is known that the direct chemical reaction of **2** and **3** with ammonia afford **1**, which would proceed through intermediates **10'** and **21**, respectively. In a similar manner, the production of **15** from **2** in the presence of hydroxylamine may be caused by the chemical reaction of **2** and hydroxylamine or by fast oxime formation. To discriminate between these possibilities, the reaction of **2** and **3** was performed in the absence of β-glucosidase, finding that the reaction with hydroxylamine hardly reached 40% in 12 h. However, an unknown side product (~8%) that could react with lactone was observed. This side product was not obtained in the presence of β-glycosidase, which indicates that the enzymatic hydrolysis is necessary to achieve high conversion.

Table 1. Enzymatic hydrolysis of **2** or **3** in the presence of hydroxylamine

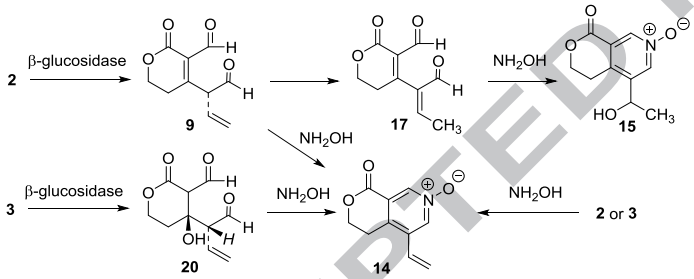


Entry	Substrate	pH	NH_2OH (equiv.)	Yield of **14** (%)	Conversion to **14**[a] (%)	Yield % of **15**(%)	Recovery (%)
1	**2**	5.5	2.4	32	33	38	3.2
2	**3**	5.5	2.4	52	62	-[b]	16
3	**3**	6.0	2.4	71	82	-[b]	12
4	**3**	6.5	2.4	41	50	-[b]	16
5	**3**	7.0	2.4	25	29	-[b]	14
6	**3**	6.0	1.0	32	71	-[b]	54
7	**3**	6.0	1.5	43	68	-[b]	36
8	**3**	6.0	2.0	65	84	-[b]	22
9	**3**	6.0	2.5	52	64	-[b]	19
10	**3**	6.0	3.0	41	49	-[b]	15

The conditions: substrate; 50 mg, 0.1 M Na-Phosphate buffer; 20 mL, incubated at 38 °C for 12 h. β-Glucosidase (0.2 mg in 200 μL buffer) was added every 1 h for 4 h. [a] Conversion was calculated on the basis of the amount of consumed substrate. [b] Not isolated.



Scheme 3 Plausible reaction pathways from **2** and **3** affording **1** and **13**



Scheme 4 Plausible routes of the enzymatic hydrolysis of **2** and **3** in the presence of hydroxylamine affording **14** and **15**

3. Conclusion

In this study, an efficient synthesis of gentianine *N*-oxide **14** was achieved in good yield (81%) by the enzymatic hydrolysis of **3**. Interestingly, the enzyme β-glycosidase was effective under conditions including hydroxylamine, and **14** was obtained as a single product. This procedure could be very useful for the synthesis of pharmaceutically important gentianine derivatives, since pyridine *N*-oxides are generally more nucleophlically reactive than pyridines.

The reaction pathways to afford **1**, **13**, **14**, and **15** from **2** and **3** were investigated in detail. The results suggest that the formation of **13** most likely involves an isomerized aglycon of **2** (**9**). This pathway provides helpful information for the study of the degradation of **2** and **3**.

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21. Starting materials **2** (5.1%) and **3** (2.1%) was isolated from Gentian root (100 g) and Swertia herb (200 g), respectively. The structure of the known compounds in this paper was determined by comparing their spectral data with those of reported value.
22. Gentianol *N*-oxide (**15**): Colorless amorphous solid. IR (neat) ν 3492(br), 3061, 2971, 1732, 1616, 1474, 1446, 1310, 1115 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) ^1H NMR ($CDCl_3$, 400 MHz); 8.67 (s, 1H), 8.58 (s, 1H), 5.13 (q, 1H, *J* = 6.8 Hz), 4.57–4.66 (m, 2H), 3.19–3.30 (m, 2H), 1.47 (t, 3H, *J* = 6.8 Hz). ^{13}C NMR (CD_3OD, 100 MHz) δ; 161.8, 142.0, 141.0, 140.9, 137.6, 125.3, 66.8, 55.5, 24.0, 23.1, 16.9. ESI-HR-MS m/z: 210.0778 (Calced for $C_{10}H_{12}NO_4$: 210.0766).
23. Gentianol acetate (**16**): yellowish amorphous solid. IR (neat) ν 2980, 2925, 1731, 1576, 1426, 1298, 1241, 1139 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ; 9.20 (s, 1H), 8.82 (s, 1H), 5.99 (q, 1H, *J* = 6.8 Hz), 4.51–4.62 (m, 2H), 3.27 (ddd, 2H, *J* = 4.5, 6.0, 16.9 Hz), 3.05 (ddd, 2H, *J* = 5.5, 6.3, 16.9 Hz), 2.08 (s, 1H), 1.59 (d, 3H, *J* = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ; 170.3, 163.5, 151.8, 151.3, 145.4, 133.8, 121.2, 67.8, 66.3, 24.0, 21.4, 21.1. ESI-HR-MS m/z: 370.2884 (Calced for $C_{12}H_{13}NO_4$: 235.0845).

Graphical Abstract

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Synthesis of Gentianine *N*-Oxide by Enzymatic Hydrolysis of Swertiamarin in the Presence of Hydroxylamine and Reaction Pathway to Gentianine and Gentianol

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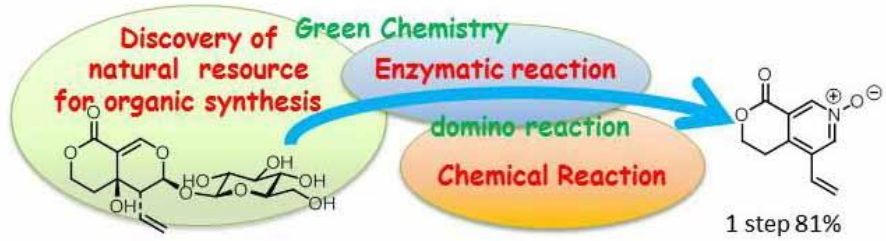

Highlights

*Swertiamarin and gentiopicroside are natural resource for organic synthesis

*The use of natural products is preferred in view of green chemistry

*The use of enzymatic reaction is preferred in view of green chemistry.

*We demonstrated a combination of enzymatic hydrolysis and oxime form

*The hydrolysis of swertiamarin afford gentianine *N*-oxide selectively