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Concise synthesis of glyconoamidines as affinity ligands for the purification of β-glucosidase involved in control of some biological events including plant leaf movement

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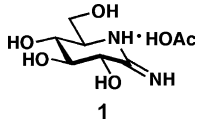
Abstract—Glycosidases are involved in deactivation or storage of some endogenous bioactive substances through biologically intriguing processes. For example, nyctinastic leaf movement is controlled by a biological clock through the regulation of β-glucosidase activity. Ganem's glyconoamidine (**1**) is used as a micromolar inhibitor of glucosidase in biochemical studies and would be useful as an affinity ligand for purification of glycosidase. However, its use for the specific inhibition of glucosidase which is highly specific to a glycoside with voluntary aglycon is seriously restricted because no universal method for the synthesis of *N*-alkylated glyconoamidine has been reported. Here, we report a concise synthesis of *N*-alkylated Ganem's glyconoamidine with voluntary aglycon using a non-protected sugar derivative.

Glycosidases are involved in deactivation or storage of some endogenous bioactive substances through biologically intriguing processes.[1] In these processes, β-glucosidase plays a key role in the control of intracellular concentration of bioactive substances to convert its β-glucoside (inactive form) into the corresponding aglycon (active form). The glycoside hydrolases are classified into 90 families according to their amino acid sequences, and each family contains a number of glycosidases with different aglycon substrate specificities. An inhibitor of glycosidase is widely used for bioorganic studies of the enzyme, and is also a good affinity ligand for the purification of glycosidase.[2] However, substrate specificity of glycosidase with respect to the aglycon moiety usually raises a serious problem in the molecular design of glycosidase inhibitor.



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An amidine-type β-glycosidase inhibitor, which was found in nature as nagstatin,[3] was developed by Ganem,[4] Tatsuta,[5] Wong,[6] Hiratake,[7] et al. Ganem's glyconoamidine (**1**) is a micromolar inhibitor of β-glucosidase. Several examples were reported on the synthesis of this type of compounds.[8–11] Most of these methods require the benzyl-protected sugar derivatives, thus some functional groups, such as olefin, cannot survive in the deprotection conditions. We developed a novel universal method for the synthesis of *N*-alkyl glyconoamidine using a non-protected sugar derivative and synthesized two inhibitors of glucosidase through the internal processes of deactivation or storage of bioactive substances in living cells.

Nyctinastic leaf movement is a typical example of a biological event in which β-glucosidase plays a key role. Most leguminous plants close their leaves in the evening, as if to sleep, and open them early in the morning according to the circadian rhythm controlled by a biological clock. Leaf movement is controlled by a change in the concentration of a glucoside-type leaf-movement factor, which is induced by the circadian rhythmic regulation of β-glycosidase activity by a biological clock.[12,13] A biological clock maintains the rhythm of leaf movement through the regulation of β-glycosidase activity. Thus, β-glucosidase is a key enzyme of nyctinasty, and

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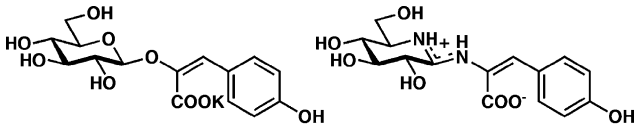
chemical studies on the rhythm of nyctinasty requires purification of the enzyme.

In *Lespedeza cuneata* Don, change in the concentration of a glucoside-type leaf-opening substance (potassium lespedezate; **2**),[14] induces the rhythm of nyctinastic leaf movement. Purification of β-glucosidase which has substrate specificity to **2** is a key step in the mechanism of the circadian rhythmic regulation of nyctinastic leaf movement. Thus, we tried to synthesize an *N*-alkyl glyconoamidine analog of **2** (**3**) which is expected to be a good ligand for the affinity chromatography of the desired β-glucosidase.



We used thionolactam (**4**), which was synthesized according to Vasella's method,[15,16] as a sugar moiety (Table 1). Non-protected thionolactam (**4**) was used in the coupling with amine (**5**) because no protected **4** could give a coupling product: when we used acetyl-protected **4**, the resulting glyconoamidine was completely decomposed under the deprotection conditions because the amidine group in the resulting glyconoamidine (**6**) was very weak under the basic conditions. And the use of benzyl or TMS-protected **4** resulted in no reaction.

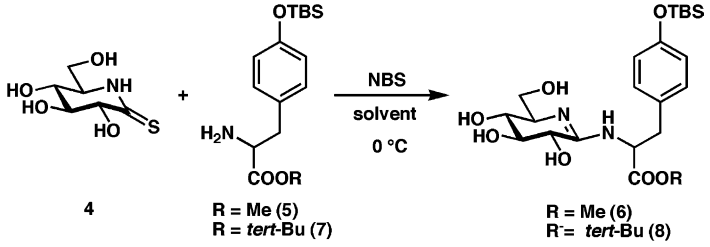
We examined the coupling of **4** with **5** under several conditions using some activators of **4**. Alkaline condition (K_2CO_3, pyridine), or the use of NBS, $Hg(OAc)_2$, and PbO gave no coupling product. Only HgO gave a small amount (16%) of a coupling product (Table 1). In these cases, the amino group of **5** would attack the carbonyl of another **5**, instead of the attack to **4**, to give an oligomer. Thus, we changed the amine from **5** into its corresponding *tert*-butyl ester (**7**) to prevent self-condensation. When **4** and **7** were coupled in pyridine–THF (9:1) by using NBS (2 equiv), **8** was obtained in 87% yield. Excess amount of **7** (ca. 10 equiv) was essential for the good yield. And the yield was strongly affected by the ratio of the mixed solvent.

Next, we examined the introduction of olefin in **8**. DDQ oxidation[14] of **8** gave a decomposed product, and direct bromination of **8** by NBS resulted in no reaction. Thus, we synthesized fluorinated aglycon **9**. Coupling of **9** with **4** and following defluorination would give the corresponding olefin. The synthesis of **9** was carried out according to Scheme 1.[17]

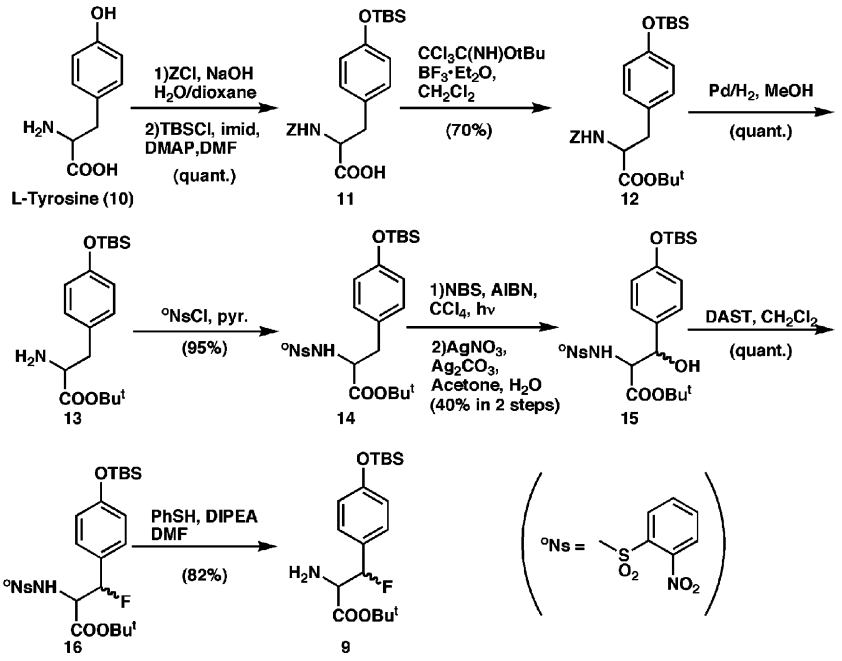
We examined coupling of **9** with **4**. When the reaction was carried out in DMF, coupling product **17** was directly obtained in 90% yield with formation of olefin and deprotection of TBS yield together with 10% of TBS-protected **17** (**18**) (Scheme 2). Compound **18** can also be converted into **17** quantitatively by the treatment with TBAF-AcOH. Long reaction time (ca. 5 h) was important for direct formation of **17**. Shorter reaction time gave intermediates, such as **18**. After purification by HPLC (Cosmosil 5C18AR column, 30% MeOHaq containing 1% AcOH), **17** was treated with TFA to give **19**[18] quantitatively. The geometry of olefin and stereochemistry of the alkyl amidine moiety in **19** was determined to be *anti*-(*Z*) by NOE experiment between H_2 and $H_{2'}$, and H3 and $H_{3'}$ (Scheme 3). The resulting **19** was photoisomerized into a 1:1 mixture of (*Z*)-**19** and (*E*)-**3**. And (*E*)-**3**[19] was isolated by HPLC (Develosil C30-HG, H_2O containing 1% AcOH). Small NOE between $H_{2'}$ and olefinic H demonstrated that the geometry of olefin in **3** is (*E*) and stereochemistry of the amidine moiety is *anti*.

Inhibitory activities of **3** and **19** against various glycosidases are shown in Table 1. In Table 1, β-glucosidase activity was determined by measuring the absorbance at 405 nm of *p*-nitrophenol formed from *p*-nitrophenyl-β-

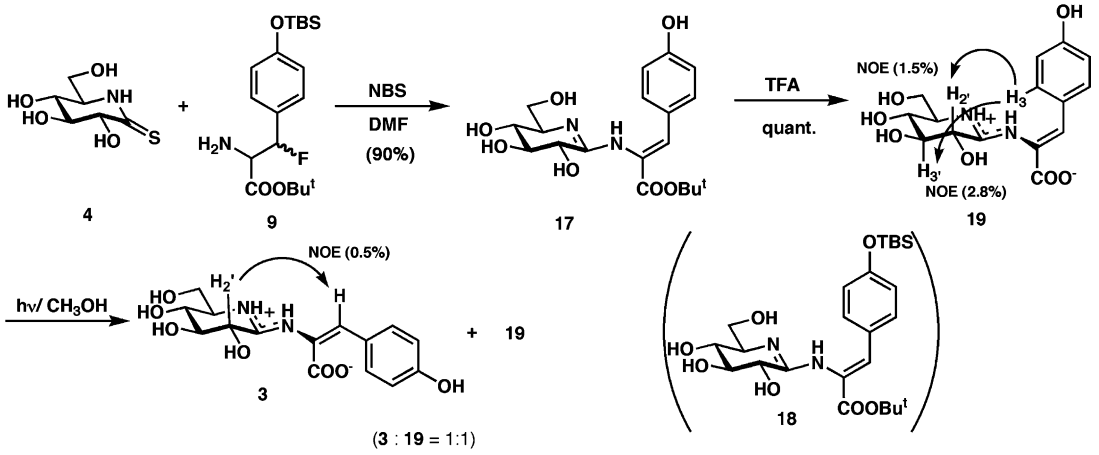
Table 1. Formation of glyconoamidine



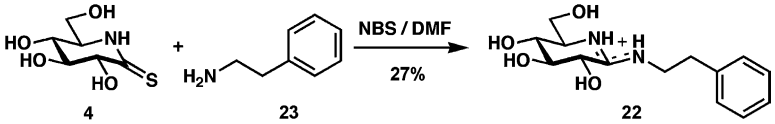
Aglycon (equiv)	Activator	Solvent	Yield (%)
5 (2 equiv)	HgO (2 equiv)	MeOH/Pyr = 9:1	16
7 (1 equiv)	NBS (2 equiv)	THF/Pyr = 1:1	9
7 (2 equiv)	NBS (2 equiv)	THF/Pyr = 1:1	38
7 (2 equiv)	NBS (2 equiv)	Pyr	Many spots
7 (10 equiv)	NBS (2 equiv)	THF/Pyr = 1:1	59
7 (2 equiv)	NBS (2 equiv)	THF/Pyr = 9:1	46
7 (10 equiv)	NBS (2 equiv)	THF/Pyr = 9:1	87



Scheme 1. Synthesis of fluorinated aglycon (**9**).



Scheme 2. Synthesis of glyconoamidine-type inhibitor of β-glycosidase (**3**) concerning the control of nyctinastic leaf movement.



Scheme 3. Synthesis of glyconoamidine-type inhibitors of biological events in plant.

D-glucopyranoside by spectroscopic method.[20] And inhibitory activities of synthetic *N*-alkyl glyconoamidines against a few enzymes were determined by interpretation of the Dixon plot.[21] K_i values against α-glucosidase and β-galactosidase were determined similarly. Interestingly, a distinct difference was observed in the K_i values of (*E*)-**3** and (*Z*)-**19** against β-glucosidase from *Aspergillus niger*. K_i value of (*Z*)-**19** was 5.8×10^{-5} M, whereas that of (*E*)-**3** was 1.6×10^{-6} M (Table 2). Inhibitory activity of **3** was 40-fold as strong as that of **19**.

Interestingly, (*E*)-**3** showed extremely high specific inhibitory activity against β-glucosidase, whereas (*Z*)-**19** inhibited β-glucosidase as strong as α-glucosidase (Table 2). Both **3** and **19** did not inhibit β-galactosidase at all. Here, we synthesized micro-molar β-glucosidase inhibitor **3**, which is expected to be a useful affinity ligand for the purification of the key enzyme controlling nyctinasty.

According to our method, various glyconoamidine-type glucosidase inhibitors can be obtained by using various

Table 2. Inhibitory activities of **3** and **19** against glycosidases

Enzyme	K_i of **3** (μM)	K_i of **19** (μM)
β-Glycosidase (*Aspergillus niger*)	1.6	58
β-Glycosidase (Almonds)	255	540
α-Glycosidase (*Bacillus* sp.)	436	52
β-Galactosidase (*Aspergillus oryzae*)	—	—

K_i values were measured at optimal pH of each enzyme [50 mM acetate buffer (pH 5.0 for β-glucosidases and pH 6.8 for α-glucosidase)].

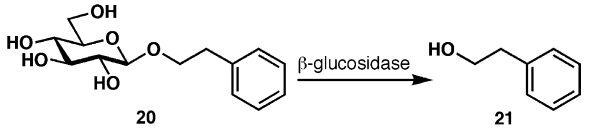


Figure 1. Release of bioactive aglycon from corresponding β-glycosides.

aglycons containing an amino group. The use of non-protected **4** would serve for both shortening of the synthetic route and expanding versatility of this reaction: applicable for a wide range of aglycons with various functional groups which would be decomposed in the deprotection conditions.

Glycoside **20**, which is stored or deactivated as a glycoside, is known to be released as an active form by the action of specific β-glucosidase. 2-Phenylethanol (**21**), that is stored as an aroma precursor of 2-phenylethyl β-D-glucopyranoside (**20**), is one of the dominant floral scent compounds emitted from roses.[22] β-Glucosidase is involved in the emission of **21** when the rose flower opens (Fig. 1). We synthesized glyconoamidines (**22**) from corresponding glucoside **20** by using phenylethylamine (**23**) and **4** by using NBS in 27% yield (Scheme 3). The yield of this coupling reaction seems to be low. However, this is because most of **22** was lost in repeated purification by silica gel or ODS chromatography even in acidic conditions. The K_i value of **22** against β-glucosidase (*Aspergillus niger*) was determined to be 1 nM according to the same method as described above. And the inhibitory activity of **22** was specific to β-glucosidase: **22** did not show any inhibitory activity against α-glucosidase (*Bacillus* sp.).

Compound **22**[23] would be a useful inhibitor of this biological event in biochemical studies of this process. Inhibitors of β-glucosidases which are specific to **20**, would be a useful tool for biochemical studies of this biological event, and can be used as a ligand in affinity chromatography for the purification of the glucosidase.

Purification of the β-glycosidase concerning nyctinasty is now in progress. Details on this enzyme will be reported in due course.

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18. **19**: ^{1}H NMR (300 MHz, CD_3OD, rt): 7.59 (1H, s), 7.43 (2H, d, J = 8.4 Hz), 6.79 (2H, d, J = 8.4 Hz), 4.32 (1H, d, J = 9.6 Hz), 3.75 (1H, dd, J = 7.2, 9.3 Hz), 3.69 (1H, dd, J = 3.0, 11.7 Hz), 3.61 (1H, dd, J = 9.3, 9.6 Hz), 3.52 (1H, dd, J = 3.6, 11.7 Hz), 3.21 (1H, ddd, J = 3.0, 3.6, 7.2 Hz) ppm; ^{13}C NMR (75 MHz, CD_3OD, rt): 170.0, 165.3, 160.6, 136.4, 132.9, 125.9, 125.5, 116.8, 74.2, 70.4, 69.7, 62.3, 61.9 ppm; HR ESI MS (positive): $[M+H]^+$ Found m/z 339.1187, $C_{15}H_{19}N_2O_7$ requires m/z 339.1192; IR (film) ν: 3207, 1668, 1606, 1558, 1514, 1379 cm^{-1}; $[\alpha]_D^{18}$ −21.6 (c 0.50, CH_3OH).
19. **3**: ^{1}H NMR (300 MHz, D_2O, rt): 7.40 (2H, d, J = 8.4 Hz), 6.88 (2H, d, J = 8.4 Hz), 6.73 (1H, s), 4.48 (1H, d, J = 8.8 Hz), 3.891 (1H, dd, J = 8.8, 9.5 Hz), 3.893 (1H, dd, J = 3.0, 12.4 Hz), 3.84 (1H, dd, J = 9.0, 9.5 Hz), 3.75

(1H, dd, $J = 4.4$, 12.4 Hz), 3.59 (1H, ddd, $J = 3.0$, 4.4, 9.0 Hz) ppm; ^{13}C NMR (75 MHz, D_2O, rt): 172.0, 166.0, 158.2, 133.0, 132.5, 128.0, 126.6, 117.0, 73.5, 70.3, 68.9, 61.7, 61.6 ppm; HR ESI MS (positive): $[M+H]^+$ Found *m/z* 339.1188, $C_{15}H_{19}N_2O_7$ requires *m/z* 339.1187; IR (film) *v*: 3246, 1664, 1609, 1560, 1514, 1411 cm^{-1}; $[\alpha]_D^{26}$ −26.1 (*c* 0.40, H_2O).

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23. **22**: ^1H NMR (300 MHz, CD_3OD, rt): 7.35–7.21 (5H, m), 4.12 (1H, d, $J = 9.6$ Hz), 3.78 (1H, dd, $J = 3.0$, 11.7 Hz), 3.72 (1H, br t, $J = 9.5$ Hz), 3.70 (1H, dd, $J = 3.5$, 11.6 Hz), 3.64–3.57 (3H, m), 3.50 (1H, br t, $J = 9.6$ Hz), 2.95 (2H, br t, $J = 7.0$ Hz); ^{13}C NMR (75 MHz, CD_3OD, 30 °C): 166.1, 139.0, 130.0, 129.8, 128.0, 74.2, 70.0, 69.4, 62.3, 61.4, 44.1, 35.0 ppm; HR ESI MS (positive): $[M+H]^+$ Found *m/z* 281.1496, $C_{14}H_{21}N_2O_4$ requires *m/z* 281.1496; IR (film) *v*: 3238, 2926, 2858, 1672, 1560, 1412 cm^{-1}; $[\alpha]_D^{25}$ +9.42 (*c* 0.10, CH_3OH).