Sulfated Purine Alkaloid Glycosides from the Pupal Case Built by the Bruchid Beetle *Bruchidius dorsalis* inside the Seed of *Gleditsia japonica*

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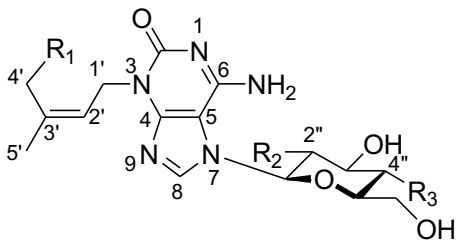
Three new sulfated isoguanine alkaloid glycosides, designated as saikachinoside A monosulfate (**1**), saikachinoside A disulfate (**2**), and locustoside B disulfate (**3**), have been isolated from the pupal case of the wild bruchid seed beetle *Bruchidius dorsalis* (Chrysomelidae, Bruchinae) infesting the seed of *Gleditsia japonica* Miquel (Fabaceae). Their structures were determined by spectroscopic methods and the inhibitory activity of **2** and **3** against acid phosphatase was evaluated.

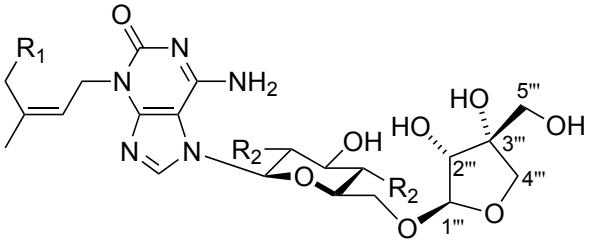
Keywords: Bruchid seed beetle, *Bruchidius dorsalis*, Pupal case, *Gleditsia japonica*, sulfated purine alkaloid.

Introduction

Larvae of the wild bruchid seed beetle *Bruchidius dorsalis* (Chrysomelidae, Bruchinae) infest dry mature seeds of the Japanese honey locust *Gleditsia japonica* Miquel (Fabaceae), and build pupal cases with their secretion/excretion products in the seeds.[1–3] Intact seeds of *G. japonica* have been reported to contain rare N^3-prenylated purine alkaloid glycosides, namely, locustosides A[4] and B[5] and saikachinosides A–C.[5][6] In the course of our studies on biologically active compounds derived from the interaction between the seed-eating larvae of the bruchid beetle *B. dorsalis* and the host plant, three rare sulfated purine alkaloid glycosides, designated as saikachinoside A monosulfate (**1**), saikachinoside A disulfate (**2**), and locustoside B disulfate (**3**), were isolated from the aqueous extract of the pupal cases. Herein, we report the purification, structural elucidation, and biological activity of the new sulfates **1**–**3**.



1 : $R_1 = R_3 = OH$, $R_2 = OSO_3H$
2 : $R_1 = OH$, $R_2 = R_3 = OSO_3H$
Locustoside A: $R_1 = H$, $R_2 = R_3 = OH$
Saikachinoside A: $R_1 = R_2 = R_3 = OH$
Saikachinoside C: R_1 = O-β-D-Glc, $R_2 = R_3 = OH$



3 : $R_1 = H$, $R_2 = OSO_3H$
Locustoside B: $R_1 = H$, $R_2 = OH$
Saikachinoside B: $R_1 = R_2 = OH$

Results and Discussion

The pupal cases were cut into small pieces and washed with hexane, EtOAc, and MeOH, successively. The residue was extracted with H_2O, and the obtained H_2O-soluble material was repeatedly separated on an octadecylsilyl (ODS) column using gradient mixtures of MeOH and H_2O to afford **1**–**3**.

Compound **1** was obtained as a colorless oil and exhibited a $[M - H]^-$ ion at *m/z* 476 in the negative-ion ESI-MS spectrum. The intensity ratio between the $[M - H]^-$ and $[M - H + 2]^-$ peaks was 100:4, indicating the presence of a sulfur atom.[7] This was confirmed by negative-ion HR-ESI-FT-MS analysis {*m/z* 476.1088 ($[M - H]^-$; calc. $C_{16}H_{22}N_5O_{10}S^-$, 476.1093)}, which established the molecular formula of **1** as $C_{16}H_{23}N_5O_{10}S$. The IR absorption bands at 3390, 3327, 1628, 1585, 1257, and 1236 cm^{-1} suggested the presence of OH, NH, C=O, C=N, C=C, and sulfate[8][9] functionalities. The presence of the sulfate group was supported by the fragment ion peak at *m/z* 396 $[M - HSO_3]^-$ in the negative-ion ESI-MS/MS spectrum (*Fig. 1*). The UV (H_2O) absorption maxima at 288 (log ε 3.81) and 246 nm (log ε 3.84) implied the presence of a 3,7-disubstituted isoguanine skeleton,[10][11] which was supported by the ^{1}H- and ^{13}C-NMR data [δ(H) 8.17 (1H, *s*, H-C(8)); δ(C) 157.6 (C(2)), 154.1 (C(4)) , 105.4 (C(5)), 154.6 (C(6)), 145.6 (C(8))] (*Table 1*). The ^{13}C-NMR spectrum also revealed the presence of two additional sp^2 carbon atoms at δ(C) 140.3 (C) and 122.6 (CH), eight heteroatom-substituted carbons at δ(C) 86.0 (CH), 80.2 (CH), 79.8 (CH), 75.6 (CH), 69.0 (CH), 61.4 (CH_2), 60.0 (CH_2), and 42.1 (CH_2), and a vinylic methyl at δ(C) 21.7 (CH_3). The presence of a 4-hydroxyisopentenyl unit was established on the basis of the HMBC correlations from δ(H) 4.74 (2H, *d*, *J* = 7.0 Hz, H_2-C(1')) to δ(C) 140.3 (C(3')) and 122.6 (C(2')), from δ(H) 5.39 (1H, *br. t*, *J* = 7.0 Hz, H-C(2')) to δ(C) 61.4 (C(4')) and 21.7 (C(5')), from δ(H) 4.27 (2H, *br. s*, H-C(4')) to δ(C) 140.3 (C(3')), 122.6 (C(2')), and 21.7 (C(5')), and from δ(H) 1.76 (3H, *br. s*, H-C(5')) to δ(C) 140.3 (C(3')), 122.6 (C(2')), and 61.4 (C(4')). Moreover, the HMBC correlations from H-C(1') to C(2) and C(4) indicated the attachment of the 4-hydroxyisopentenyl unit to *N*-3 of the isoguanine unit (*Fig. 2*). The *Z* geometry of $\Delta^{2'}$ of the 4-hydroxyisopentenyl moiety was suggested by comparison of the NMR chemical shifts of H-C(5') (δ(H) 1.76) and C(5') (δ(C) 21.7) with those reported in the literature (for *Z* geometry: δ(H) 1.79 and δ(C) 21.1[12]; for *E* geometry: δ(H) 1.80 and δ(C) 13.8[13]), and was confirmed by difference NOE experiments, which showed an enhancement of the olefinic proton (H-C(2')) upon irradiation of the methyl protons (H-C(5')) (*Fig. 2*). Because of the overlap of some oxygenated methine signals in the 3.8–3.9 ppm region of the ^{1}H-NMR spectrum (MeOH-d_4–D_2O, 1:9), the NMR spectra recorded in acetone-d_6–D_2O (4:1) were also used for further structural analysis. Interpretation of ^{1}H,^{1}H-COSY data in conjunction with vicinal coupling constants led to a β-glucopyranosyl unit (C(1'')–C(6'')). HMBC correlations from H-C(1'') to C(8) and C(5) of the isoguanine unit indicated the attachment of the β-glucopyranosyl unit to *N*-7 of the isoguanine unit (*Fig. 2*). This was confirmed by an NOE experiment in which irradiation of H-C(8) caused an enhancement of the anomeric proton (H-C(1'')) (*Fig. 2*). The ^{1}H- and ^{13}C-NMR spectra were similar to those reported for saikachinoside A,[6] except that the signals of C(2'') of the β-glucopyranosyl unit in the spectrum of **1** were shifted downfield from δ(H) 3.70 and δ(C) 73.2 to δ(H) 4.31 and δ(C) 79.8, respectively, indicating the attachment of the *O*-sulfate group to C(2''). This was supported by the fragment ion peaks at *m/z* 234 $[M - 243]^-$ and 241 $[M - 236]^-$ in the negative-ion MS/MS spectrum (*Fig. 1*). On acid-catalyzed desulfation with trifluoroacetic acid (TFA), compound **1** afforded saikachinoside A,[6] indicating that the absolute configuration of the 2''-*O*-sulfated glucosyl unit in **1** is D. Hence, compound **1** is 7-(β-D-2-sulfonyloxyglucopyranosyl)-3-[(*Z*)-4-hydroxy-3-methyl-2-butenyl]isoguanine, which has been assigned the trivial name saikachinoside A monosulfate.

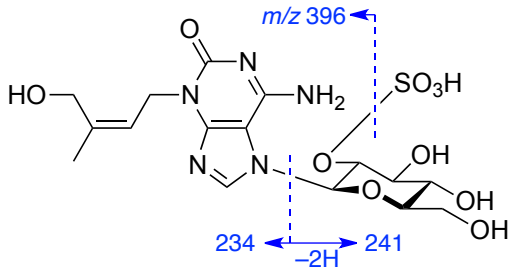


Figure 1. Negative-ion ESI-MS/MS analysis of **1**.

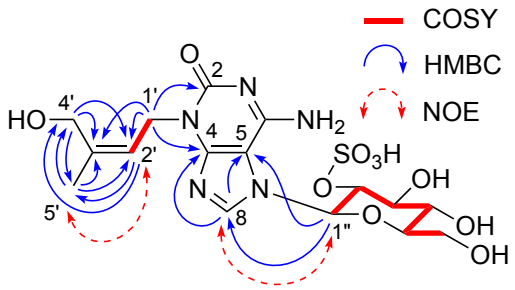


Figure 2. Key ^{1}H,^{1}H-COSY, HMBC, and NOE correlations for **1**.

Compound **2** was obtained as a colorless oil, and its molecular formula was determined to be $C_{16}H_{23}N_5O_{13}S_2$ by negative-ion HR-ESI-FT-MS analysis {*m/z* 556.0664 ([*M* – H]$^-$; calc. $C_{16}H_{22}N_5O_{13}S_2^-$, 556.0661)}. The divalent ion at *m/z* 277.5298 ([*M* – 2H]$^{2-}$; calc. $C_{16}H_{21}N_5O_{13}S_2^{2-}$/2, 277.5294)} in the HR-ESI-FT-MS spectrum indicated the presence of two sulfate groups. The 1D and 2D NMR spectra were very similar to those of **1** (*Table 1* and *Fig. 3*), except that the ^{1}H and ^{13}C signals of C(4'') of the glucosyl unit in the spectrum of **2** were shifted downfield from δ(H) 3.84 and δ(C) 69.0 to δ(H) 4.59 and δ(C) 75.4, respectively, indicating the attachment of an additional *O*-sulfate group to C(4''). This was supported by ESI-MS/MS data (*Fig. 4*). The geometry of the double bond was assigned as *Z* on the basis of the NOE correlation between the methyl protons (H-C(5')) and the olefinic proton (H-C(2')) (*Fig. 3*). On acid-catalyzed desulfation with TFA, compound **2** afforded saikachinoside A, indicating that the absolute configuration of the 2'',4''-di-*O*-sulfated glucosyl unit in **2** is also D. Hence, compound **2** is 7-[β-D-2,4-bis(sulfonyloxy)glucopyranosyl]-3-[(*Z*)-4-hydroxy-3-methyl-2-butenyl]isoguanine, which has been assigned the trivial name saikachinoside A disulfate.

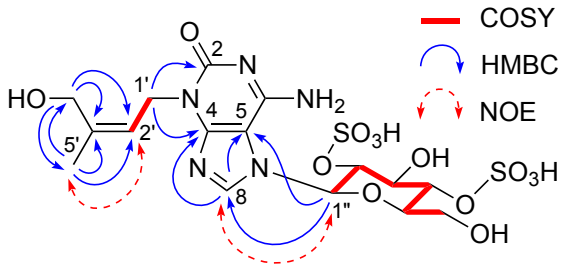


Figure 3. Key ^{1}H,^{1}H-COSY, HMBC, and NOE correlations for **2**.

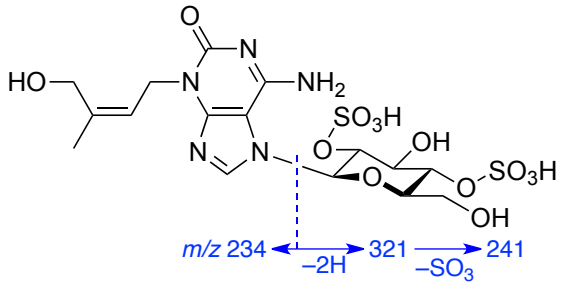


Figure 4. Negative-ion ESI-MS/MS analysis of **2**.

Compound **3** was obtained as a colorless oil, and its molecular formula was established as $C_{21}H_{31}N_5O_{16}S_2$ by negative-ion HR-ESI-FT-MS analysis {*m/z* 672.1136 ([*M* – H]$^-$; calc. $C_{21}H_{30}N_5O_{16}S_2^-$, 672.1134)}. The HR-ESI-FT-MS of **3** also showed a divalent ion at *m/z* 335.5532 ([*M* – 2H]$^{2-}$; calc. $C_{21}H_{29}N_5O_{16}S_2^{2-}$/2, 335.5531), indicating the presence of two sulfate groups. The ^{1}H- and ^{13}C-NMR spectra were similar to those of **2** (*Table 1*), except for additional signals attributable to a pentosyl and a vinylic methyl moiety, and the absence of the oxygenated methylene signal of the N^3-prenyl group. Because of the overlap of some oxygenated methine and methylene signals in the 3.8–5.2 ppm region of the ^{1}H-NMR spectrum (MeOH-d_4–D_2O, 1:9), the NMR spectra recorded in DMSO-d_6 were also used for further structural analysis. On the basis of a comparison of the ^{13}C-NMR chemical shifts for the pentosyl moiety with those previously reported in

the literature[5][14] and 2D NMR analysis (*Fig. 5*), the pentose was identified as β-apiofuranose. The HMBC correlation from the anomeric proton (H-C(1‴)) of the β-apiofuranosyl unit to the oxygenated methylene carbon (C(6")) of the glucopyranosyl unit and the downfield chemical shift of C(6") of the glucopyranosyl unit indicated the attachment of the β-apiofuranosyl unit to C(6") of the glucopyranose. This was supported by the NOESY correlation between H-C(1‴) and H-C(6") (*Fig. 5*). Moreover, the ^{1}H and ^{13}C chemical shifts of C(2") and C(4") of the glucosyl unit of **3** were similar to those of **2**, indicating that the *O*-sulfate groups were attached to C(2") and C(4") as in **2**. The presence of the di-*O*-sulfated disaccharide unit was supported by the fragment ion peaks at *m/z* 453, 373, and 218 in the negative-ion ESI-MS/MS spectrum (*Fig. 6*). All other HMBC correlations also supported that compound **3** is the 2",4"-di-*O*-sulfated analogue of locustoside B[5] isolated from the seed of *G. japonica* (*Fig. 5*). Attempted acid-catalyzed desulfation of **3** with TFA resulted in the formation of locustoside A[4] (as determined by ESI-FT-MS) *via* hydrolysis of the apiosyl unit as well as desulfation of **3**. Base-catalyzed desulfation of **3**, however, afforded locustoside B, indicating the absolute configuration of **3** to be the same as that of locustoside B. Hence, compound **3** is 7-[*β*-D-apiofuranosyl-(1–>6)-*β*-D-2,4-bis(sulfonyloxy)glucopyranosyl]-3-(3-methyl-2-butenyl)-isoguanine, which has been assigned the trivial name locustoside B disulfate.

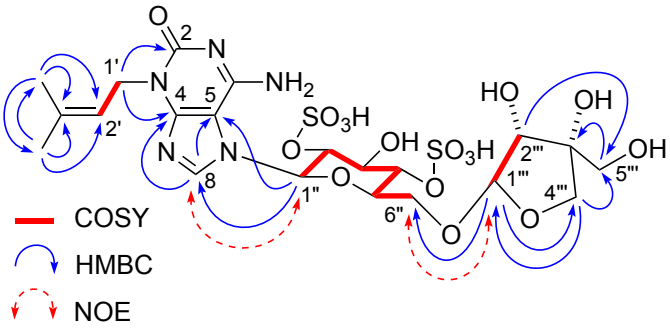


Figure 5. Key $^{1}H,^{1}H$-COSY, HMBC, and NOE correlations for **3**.

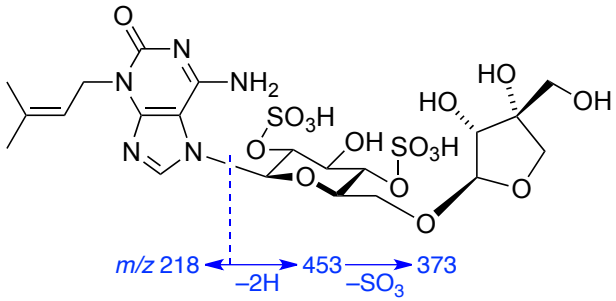


Figure 6. Negative-ion ESI-MS/MS analysis of **3**.

Table 1. ^{1}H- and ^{13}C-NMR data (400 and 100 MHz, resp.; in MeOH-d_4–D_2O, 1:9) of **1**–**3.** δ in ppm, *J* in Hz

position	**1**		**2**		**3**	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
2	157.6		152.5		156.7	
4	154.1		154.7		154.2	
5	105.4		104.2		105.2	
6	154.6		151.1		153.7	
8	145.6	8.17 (*s*)	147.4	8.33 (*s*)	145.7	8.19 (*s*)
1'	42.1	4.74 (*d*, *J* = 7.0)	41.9	4.74 (*d*, *J* = 6.7)	42.5	4.61 (*d*, *J* = 6.4)
2'	122.6	5.39 (*br. t*, *J* = 7.0)	121.2	5.39 (*br. t*, *J* = 6.7)	118.5	5.13 (*br. t*, *J* = 6.4)
3′	140.3		140.9		139.0	
4′	61.4	4.27 (*br. s*)	61.1	4.27 (*br. s*)	18.4	1.72 (*br. s*)
5'	21.7	1.76 (*br. s*)	21.4	1.76 (*br. s*)	25.7	1.60 (*br. s*)
1"	86.0	5.82 (*d*, *J* = 9.2)	85.6	5.92 (*d*, *J* = 9.2)	85.6	5.81 (*d*, *J* = 9.2)

2''	79.8	4.31 (*t*, *J* = 9.2)	79.1	4.29 (*t*, *J* = 9.2)	78.9	4.37 (*t*, *J* = 9.2)
3''	75.6	3.86 (*t*, *J* = 9.2)	73.6	4.08 (*t*, *J* = 9.2)	74.0	4.05 (*dd*, *J* = 9.5, 9.2)
4''	69.0	3.81 – 3.87 (*m*)	75.4	4.59 (*dd*, *J* = 9.8, 9.2)	75.6	4.70 (*t*, *J* = 9.5)
5''	80.2	3.80 – 3.86 (*m*)	78.7	4.01 (*ddd*, *J* = 9.8, 2.4, 1.8)	77.0	4.04 (*br. d*, *J* = 9.5)
6''	60.0	3.96 (*br. d*, *J* = 12.8)	59.7	4.05 (*dd*, *J* = 12.5, 2.4)	65.9	4.01 (*br. d*, *J* = 11.0)
		3.91 (*br. d*, *J* = 12.8)		3.94 (*dd*, *J* = 12.5, 1.8)		3.92 (*br. d*, *J* = 11.0)
1'''					110.0	5.11 (*d*, *J* = 3.1)
2'''					77.7	4.07 (*d*, *J* = 3.1)
3'''					80.3	
4'''					74.4	3.98 (*d*, *J* = 10.4)
						3.84 (*d*, *J* = 10.4)
5'''					63.7	3.64 (*br. s*)

Acid phosphatases are widely distributed in nature and appear to be ubiquitous. Several purine analogues including locustoside B have been reported to modulate acid phosphatase activity.[5][15 – 17] Whereas compound **1** could not be subjected to bioassay because of the limited amount available, the other isolated compounds were evaluated for their inhibitory activity against acid phosphatase. Compounds **2** and **3** exhibited 31.3 and 61.1% inhibitory activities, respectively, against acid phosphatase at 1 mM (Table 2). The corresponding values for saikachinoside A and locustoside B isolated from the seed of the host plant *G. japonica* were 0.0 and 23.9%, respectively.[5] The IC_{50} value of compound **3** was 0.83 mM. These findings suggest that the sulfate groups could act as phosphate mimics[18][19] to enhance the inhibitory effects on acid phosphatase.

Table 2. Acid phosphatase inhibitory activity

Compound	% Inhibition at 1 mM[a]	IC_{50} (mM)
2	31.3 ± 3.4[c]	>1.0
3	61.1 ± 1.1[c]	0.83
Saikachinoside A	0.0 ± 0.5[d]	>1.0
Locustoside B	23.9 ± 2.9[c] [d]	>1.0
Guanine[b]	16.1 ± 0.1[c]	>1.0

[a] Data are expressed as means ± SD (*n* = 2), [b] Positive control, [c] Significant differences with respect to negative control ($P < 0.01$), [d] Ref[5].

Conclusions

Three new sulfated isoguanine alkaloid glycosides named saikachinoside A monosulfate (**1**), saikachinoside A disulfate (**2**), and locustoside B disulfate (**3**) were isolated from the pupal case produced by the seed-eating larva of the bruchid beetle *B. dorsalis* inside the seed of *G. japonica*. Thus far, sulfated nucleoside derivatives, such as the kainate receptor inhibitor HF-7, have been isolated from the venom of spiders.[20][21] To the best of our knowledge, this is the first isolation of sulfated purine alkaloid glycosides from other natural sources than spiders. Since the sulfates **1**–**3** are not present in the intact seeds of *G. japonica*, they could be proposed to result from sulfation of the purine alkaloid glycosides in the seed of *G. japonica* by *B. dorsalis*. The sulfation was regiospecifically occurred at the C(2'') and/or C(4'') position(s) of the glucosyl unit of the purine alkaloid glycosides. Compounds **2** and **3** exhibited inhibitory activities against acid phosphatase. Plant acid phosphatases have been suggested to be involved at various cellular metabolic and bioenergetic levels, and to be also induced under various environmental conditions including seed germination and pathogen infection.[22][23] Moreover, it has been reported that parasitoid wasps attack larvae of bruchid beetles,[24] and acid phosphatase is a common component in venom of parasitoids.[25] Although further biological evaluation of the compounds is necessary, the ability of the larva of *B. dorsalis* to sulfate the seed constituents might be related to self-defense of the bruchid beetle against parasitoid wasps or the host plant.

Experimental Section

General

Column chromatography (CC): Wakogel 50C18 (*Wako Pure Chemical Industries, Ltd*, Osaka, Japan). Thin layer chromatography (TLC): precoated silica gel RP-18 F_{254}S plates (*Merck*, Darmstadt, Germany). Optical rotation: *JASCO P-2200* polarimeter. UV Spectra: *JASCO V-630* spectrometer; λ_{max} (log ε) in nm; IR Spectra: *JASCO FT/IR-6300* spectrometer; *v* in cm^{-1}; ECD Spectra: *JASCO J-725* spectropolarimeter; λ_{max} (Δε) in nm. ^{1}H- and ^{13}C-NMR spectra: *JEOL A400* spectrometer (400 and 100 MHz, resp.); δ in ppm rel. to residual solvent peak (δ(H) 3.30 and δ(C) 49.0 for MeOH-d_4 in D_2O; δ(H) 2.05 and δ(C) 29.8 for acetone-d_6 in D_2O; δ(H) 2.49 and δ(C) 39.5 for DMSO-d_6), *J* in Hz. HR-ESI-FT-MS: *Thermo Fisher Scientific LTQ Orbitrap XL* mass spectrometer at the Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University; in *m/z*.

Plant and insect materials

The seeds were collected from seven trees of *Gleditsia japonica* Miquel (Fabaceae) at Nagahama City (coordinates 35.3574° N, 136.2782° E) in Shiga Prefecture, Japan, and identified as described in the literature.[4][5] The seeds were kept in plastic cases until adults of *Bruchidius dorsalis* (Chrysomelidae, Bruchinae) emerged from infested seeds. Empty pupal cases were harvested after adult emergence. Voucher specimens of *B. dorsalis* (registry number HUM-Ins-0004263) and the infested seed of *G. japonica* (registry number HUM-PL-00003) have been deposited at the Hiroshima University Museum, Japan.

Extraction and Isolation

Dry pupal cases (127 g) derived from *ca*. 6,400 infested seeds were macerated and percolated with hexane, AcOEt, and MeOH successively to remove wax, and were extracted with 3 x 400 ml H_2O at room temperature for one day each, assisted by sonication. The combined aqueous layer was lyophilized to yield a syrup. A portion (1.5 g) of the H_2O-soluble material (13 g) was subjected to medium pressure CC on ODS with gradient mixtures of MeOH–H_2O (0:100 to 100:0). The first fraction (100% H_2O, 83 mg) was separated on ODS column using H_2O as an eluent to afford **1** (5 mg) and **2** (33 mg). The fourth fraction (15% MeOH, 14 mg) was purified by CC on ODS using H_2O as an eluent to afford **3** (12 mg).

Saikachinoside A monosulfate (= **7-(β-D-2-sulfonyloxyglucopyranosyl)-3-[(*Z*)-4-hydroxy-3-methyl-2-butenyl]isoguanine**; **1**). Colorless oil. $[\alpha]^{25}_D$ = +8.3 (*c* = 0.06, H_2O); UV (H_2O): 288 (3.81), 246 (43.84). IR (film): 3390, 3327, 1628, 1585, 1257, 1236. ECD (H_2O): 224 (–5.3), 245 (+1.3), 288 (+0.5). ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-FT-MS (neg.): 476.1088 ([*M* – H]$^-$, $C_{16}H_{22}N_5O_{10}S^-$; calc. 476.1093).

Saikachinoside A disulfate (= **7-[β-D-2,4-bis(sulfonyloxy)glucopyranosyl]-3-[(*Z*)-4-hydroxy-3-methyl-2-butenyl]isoguanine**; **2**). Colorless oil. $[\alpha]^{25}_D$ = +28.2 (*c* = 1.7, H_2O); UV (H_2O): 289 (3.82), 246 (3.82). IR (film): 3392, 3336, 3253, 1632, 1583, 1259, 1234. ECD (H_2O): 219 (–4.5), 246 (+1.3), 284 (+0.5). ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-FT-MS (neg.): 556.0664 ([*M* – H]$^-$, $C_{16}H_{22}N_5O_{13}S_2^-$; calc. 556.0661), 277.5298 [*M* – 2H]$^{2-}$, $C_{16}H_{21}N_5O_{13}S_2^{2-}$/2; calc. 277.5294).

Locustoside B disulfate (= **7-[β-D-apiofuranosyl-(1–>6)-β-D-2,4-bis(sulfonyloxy)glucopyranosyl]-3-(3-methyl-2-butenyl)-isoguanine**; **3**). Colorless oil. $[\alpha]^{25}_D$ = –6.5 (*c* = 0.4, MeOH–H_2O, 7:3). UV (H_2O): 291 (3.86), 245 (3.82). IR (film): 3396, 3328, 3256, 1633, 1591, 1255, 1231. ECD (H_2O): 224 (–7.9), 247 (+1.9), 288 (+0.8). ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-FT-MS (neg.): 672.1136 ([*M* – H]$^-$, $C_{21}H_{30}N_5O_{16}S_2^-$; calc. 672.1134), 335.5532 [*M* – 2H]$^{2-}$, $C_{21}H_{29}N_5O_{16}S_2^{2-}$/2; calc. 335.5531).

*Desulfation of **1***

Compound **1** (2 mg) was dissolved in D_2O (0.2 ml) and the ^{1}H-NMR spectrum was recorded. After addition of TFA (0.1 µl), the reaction mixture was heated at 60 ˚C and the reaction was monitored by ^{1}H-NMR. After 24 h of reaction at 60 ˚C, the mixture was cooled to room temperature, evaporated to dryness, and then purified by CC (ODS, CH_3CN-H_2O, CH_3CN: 0 to 10%) to afford the pure desulfated product (1 mg), which was identified as saikachinoside A[6] on the basis of its UV, ECD, and ^{1}H-NMR spectra and co-HPLC analysis. HR-ESI-FT-MS (pos.): 398.1670 ([*M* + H]$^+$, $C_{16}H_{24}N_5O_7^+$; calc. 398.1670).

Desulfation of ***2***

Compound **2** (1 mg) was treated in a similar manner as **1**, except for the reagent quantity [TFA (0.4 µl)] and reaction time (2 days). The reaction gave the desulfated product (0.5 mg), which was identified as saikachinoside A[6] on the basis of its UV, ECD, and ^{1}H-NMR spectra and co-HPLC analysis. HR-ESI-FT-MS (pos.): 398.1669 ([*M* + H]$^+$, $C_{16}H_{24}N_5O_7^+$; calc. 398.1670).

Desulfation of ***3***

A solution of compound **3** (2 mg) in pyridine-d_5 (0.5 ml) in an NMR tube was heated at 60 ˚C for 5 days. The ^{1}H-NMR spectrum of the reaction solution showed the production of the corresponding desulfated derivative. After cooling to room temperature, the reaction mixture was subjected to ODS CC using CH_3CN-H_2O (CH_3CN: 0 to 10%) to yield the pure desulfated product (1 mg), which was identified as locustoside B[5] on the basis of its UV, ECD, and ^{1}H-NMR spectra and co-HPLC analysis with the authentic sample. HR-ESI-FT-MS (pos.): 514.2136 ([*M* + H]$^+$, $C_{21}H_{32}N_5O_{10}^+$; calc. 514.2144).

Acid Phosphatase Inhibition Assay

Acid phosphatase from wheat germ was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The enzyme activity was measured according to the reported method with a slight modification.[15] Briefly, an aqueous solution of a test compound (10 mM, 10 µl) was added to a 0.1 mg/ml enzyme solution in 0.1 M sodium acetate buffer at pH 5.0 (80 µl), and preincubated for 10 min at room temperature. The enzymatic reaction was initiated by the addition of 10 µl of 2 mM *p*-nitrophenyl phosphate (*p*-NPP) as the substrate. After 15-min incubation at 37 ˚C, the reaction was terminated by the addition of 100 µl of 1 M Na_2CO_3 and the absorbance of released *p*-nitrophenolate was measured at 415 nm using a 96-well microplate reader (iMark, Bio-Rad, USA). The inactive mixture prepared by adding Na_2CO_3 prior to *p*-NPP was used as a blank, guanine[15] (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a positive control, and H_2O was used as a negative control. All experiments were performed in duplicate. Results are expressed as means ± standard deviation (SD) of percentage decrease with respect to the negative control values. Statistical differences were evaluated by Student's *t* test.

For calculating IC_{50}, inhibition assays were performed with the test compound diluted around the estimated IC_{50} value. IC_{50} was calculated from the non-linear curve fitting of percent inhibition (% inhibition) vs. inhibitor concentration.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

Author Contribution Statement

Y. H., *K. M.*, *E. O.*, *H. K.*, and *A. I.-J.*: performed the experiments; *T. N.* and *H. O.*: contributed NMR analysis; *S. O.*: conceived and designed the experiments and edited the manuscript.

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