Glucosylquestiomycin, a Novel Antibiotic from *Microbispora* sp. TP-A0184: Fermentation, Isolation, Structure Determination, Synthesis and Biological Activities

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(Received for publication July 10, 1998)

Glucosylquestiomycin, a novel *N*-glucopyranoside of questiomycin A, was isolated from the culture broth of *Microbispora* sp. TP-A0184. The absolute configuration of the sugar was determined as D-configuration by chemical synthesis. The new antibiotic showed antibacterial activity against Gram-positive and -negative bacteria and yeasts and cytotoxic activity against U937 cells.

In our screening program for new antibiotics from rare actinomycetes, we found a novel *N*-glucopyranoside of questiomycin A (**1**) along with the known questiomycin A (**2**) in the fermentation broth of *Microbispora* sp. TP-A0184, which was isolated from a soil sample collected at Kureha, Toyama, Japan. Questiomycin A has been isolated as an antibacterial substance from actinomycetes[1,2] and as a phytotoxin from a fungus[3]. The new antibiotic was glycosylated on the amino group which was more basic than those seen in carbazole glycosides such as rebeccamycin. In this paper, we report the fermentation, isolation, structure determination, synthesis and biological activities of glucosylquestiomycin (**1**).

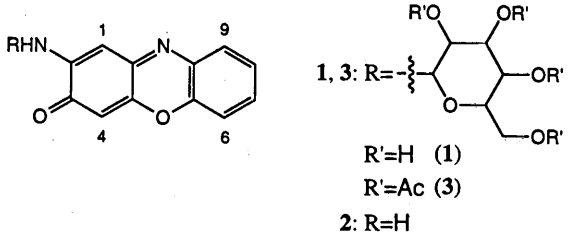
Fermentation

A loopful of a mature slant culture of TP-A0184 was inoculated into a seed medium containing 100 ml of soluble starch 1.0%, glucose 0.5%, NZ-case (Humco) 0.3%, yeast extract (Difco) 0.2%, tryptone 0.5%, KH_2PO_4 0.1%, $MgSO_4$ 0.05% and $CaCO_3$ 0.3% (pH 7.0) in a 500 ml K-1 flask and cultured at 30°C for 4 days on a rotary shaker at 200 rpm. The seed culture (1 ml each) was transferred to fifty 500 ml K-1 flasks containing 100 ml of a producing medium composed of glucose 2.0%, Protein S 1.5%, $CaCO_3$ 0.3% and NaI 0.00025% (pH 7.0) and incubated at 30°C for 8 days on a rotary shaker at 200 rpm.

Isolation

The fermentation broth (5 liters) was centrifuged to separate the mycelial cake. The supernatant was applied to a Diaion HP-20 (Mitsubishi kasei) column. The column was washed with 60% methanol and eluted with methanol. The eluate was concentrated *in vacuo* and the

Fig. 1. Structures of glucosylquestiomycin (**1**), questiomycin A (**2**) and tetra-*O*-acetate (**3**) of **1**.



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residue was chromatographed on an ODS column (YMC, ODS-A) with acetonitrile-0.15% KH_2PO_4 (pH 3.5) (7:3) to give two active fractions containing **1** (Fraction I) and **2** (Fraction II). Each fraction was further purified separately by Toyopearl HW-40F column chromatography, which was eluted with 80% methanol to give glucosylquestiomycin (**1**) (4.0 mg) and questiomycin A (**2**) (13.6 mg).

Physico-chemical Properties

The physico-chemical properties of glucosylquestiomycin (**1**) are summarized in Table 1. **1** was obtained as a red powder soluble in dimethylsulfoxide and pyridine, but insoluble in chloroform and ethyl acetate. The UV and visible spectrum showed the presence of the chromophore identical with that of questiomycin A. In IR spectrum, bands at 1590 and 3350 cm^{-1} indicated the presence of the carbonyl group and the hydroxyl group,

Table 1. Physico-chemical properties of glucosylquestiomycin.

Appearance	Red powder
MP	>195°C (dec)
$[\alpha]_D^{28}$	+16.5 (c=0.1, pyridine)
HRFAB-MS	
Found:	375.1208 $(M+H)^+$
Calcd:	375.1192 (for $C_{18}H_{19}N_2O_7$)
Molecular formula	$C_{18}H_{18}N_2O_7$
UV λ_{max}^{MeOH} nm (log ε)	240 (4.33), 423 (4.23)
IR ν_{max} (cm^{-1})	3350, 1590
Solubility	
soluble in	DMSO, pyridine
slightly soluble in	MeOH
TLC (Rf)[a]	0.34

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$-MeOH=5:1)

Table 2. ^1H and ^{13}C NMR data for glucosylquestiomycin (**1**) and related compounds.

	1 (pyridine-d_5)		**2** (DMSO-d_6)		**3** ($CDCl_3$)	
Position	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
1	101.12	6.98 (s)	98.37	6.37 (s)	101.32	6.44 (s)
2	145.99		148.70		143.90	
3	180.59		180.00		179.40	
4	104.08	6.50 (s)	103.27	6.33 (s)	104.22	6.41 (s)
4a	149.53		148.13		149.33	
5a	142.91		141.80		142.92	
6	116.27	7.27-7.32 (m)	115.71	7.45 (dd, J=1.9, 8.3 Hz)	116.10	7.39 (d, J=7.3 Hz)
7	129.70	7.35 (dt, J=1.4, 8.0 Hz)	128.60	7.44 (dd, J=1.5, 8.0 Hz)	130.24	7.48 (dd, J=1.4, 7.8 Hz)
8	125.48	7.27-7.32 (m)	125.04	7.37 (ddd, J=2.2, 6.6, 7.8 Hz)	125.39	7.38 (t, J=7.8 Hz)
9	129.17	7.82 (dd, J=1.4, 7.8 Hz)	127.81	7.68 (dd, J=1.5, 7.6 Hz)	128.91	7.74 (dt, J=1.4, 7.8 Hz)
9a	134.59		133.59		133.75	
10a	149.10		147.15		148.46	
1'	85.01	5.29 (t, J=8.3 Hz)			82.31	4.85 (t, J=9.0 Hz)
2'	74.38	4.25 (t, J=8.6 Hz)			70.65	5.20 (t, J=9.3 Hz)
3'	79.29	4.36 (t, J=8.6 Hz)			72.76	5.40 (t, J=9.5 Hz)
4'	71.50	4.31 (t, J=8.6 Hz)			68.36	5.14 (t, J=9.5 Hz)
5'	80.03	4.10 (m)			73.11	3.89 (ddd, J=2.0, 5.2, 9.5 Hz)
6'	62.52	4.38 (dd, J=2.4, 12.0 Hz)			61.80	4.18 (dd, J=2.0, 12.4 Hz)
		4.51 (dd, J=5.1, 12.0 Hz)				4.31 (dd, J=5.2, 12.4 Hz)
2-NH		7.64 (d, J=7.1 Hz)				6.59 (d, J=7.8 Hz)
-O*C*CH_3					169.43	
					170.03	
					170.50	
					170.63	
-OC*C*H_3					20.54	2.06 (s)
					20.54	2.07 (s)
					20.56	2.08 (s)
					20.66	2.12 (s)

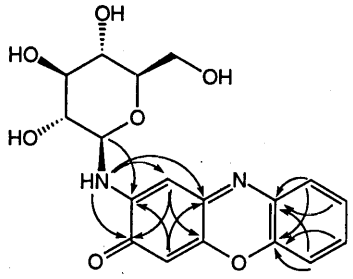
^1H and ^{13}C NMR spectra were measured at 400 MHz and 100 MHz respectively.

respectively. The high resolution FAB-MS gave a parent ion peak at m/z 375.1208 [$(M+H)^+$; calcd for $C_{18}H_{19}N_2O_7$, 375.1192]. The molecular formula of **1** was thus determined to be $C_{18}H_{18}N_2O_7$ on the basis of MS, ^1H and ^{13}C NMR spectra.

Structure Determination

The structure of questiomycin A (**2**) was confirmed by comparing the ^1H and ^{13}C NMR data with those of a reference sample, which was prepared according to the reported procedure[4]. The ^1H and ^{13}C NMR spectra (pyridine-d_5) of **1** exhibited 14 proton and 18 carbon signals, respectively, as summarized in Table 2. ^1H-^{13}C long range couplings revealed by PFG-HMBC experiment (duration time = 80 msec) are shown in Fig. 2. By comparing the ^{13}C NMR data with those of questiomycin A, 12 carbons were assignable to the questiomycin A skelton. The correlation between H-6, H-7, H-8 and H-9 confirmed by spin decoupling experiments and the long-range couplings from H-6 and H-7 to C-5a and H-8 and H-9 to C-9a showed the presence of 1,2-disubstituted benzene ring. Long-range couplings were also observed from H-1 to C-3 and C-4a and H-4 to C-2 and C-10a. In addition, an NH proton at 7.6 ppm was long-range coupled to C-1, C-3 and C-10a. These results established that the NH group was substituted at C-2 and the chromophore was identical with that of questiomycin A. The NH proton was sequentially coupled through H-1′ to H-6′, suggesting that 1-aminohexose was connected at C-2. The structure of the hexose was determined as β-glucopyranoside since the $J_{1',2'}$, $J_{2',3'}$, $J_{3',4'}$ and $J_{4',5'}$ were in the range of 8.3 ~ 8.6 Hz and the $J_{NH,1'}$ was 7.1 Hz. Therefore the structure of **1** was determined as 2-(β-glucopyranosylamino)-3*H*-phenoxazin-3-one. The proposed structure was further confirmed by the NMR analysis of 2′,3′,4′,6′-tetra-*O*-acetyl derivative (**3**), which was obtained by acetylation of **1** in pyridine with acetic anhydride.

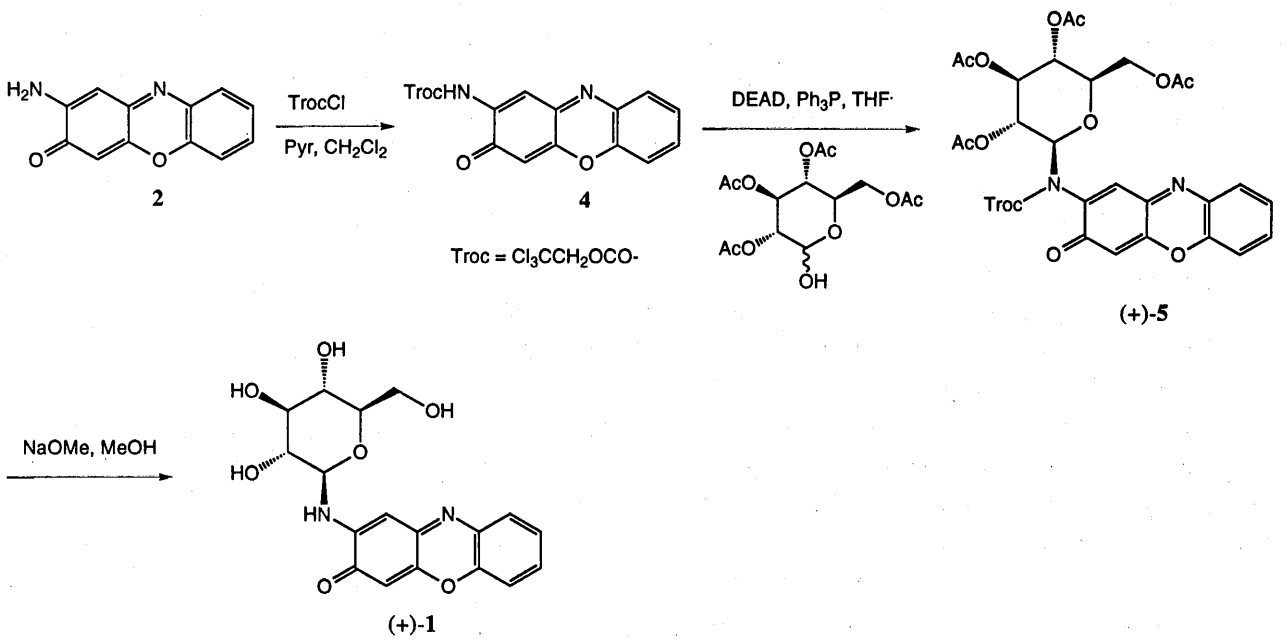
Fig. 2. ^1H-^{13}C long range couplings observed in the HMBC spectrum on glucosylquestiomycin (**1**).



Synthesis

The synthesis started from questiomycin A (**2**), which was obtained by heating *o*-aminophenol and *p*-quinone in ethanol in 30 ~ 40% yield. Nucleophilic substitution on the amino group with electrophilic reagents such as glucosyl halides and glucal epoxide was unsuccessful due to the low reactivity; therefore we examined the Mitsunobu reaction[5] to form the *N*-glycosidic linkage. To enhance the acidity of the NH group, **2** was converted

Scheme 1. Synthesis of D-(+)-glucosylquestiomycin.



2 **4** (+)-**5** (+)-**1**

Table 3. *In vitro* antibacterial activities of D- and L-glucosylquestiomycin and questiomycin A.

Organism	MIC (μg/ml)		
	D-Glucosyl-questiomycin	L-Glucosyl-questiomycin	Questiomycin A
Staphylococcus aureus 209P JC-1	>100	>100	25.0
Bacillus subtilis ATCC 6633	50.0	>100	12.5
Bacillus subtilis 122	12.5	>100	12.5
Micrococcus luteus ATCC 9341	>100	>100	25.0
Escherichia coli NIHJ JC-2	>100	>100	25.0
Pseudomonas alcaligenes JCM 5967	25.0	25.0	12.5
Pseudomonas aeruginosa A3	12.5	12.5	12.5
Saccharomyces cerevisiae S-100	6.25	6.25	1.56
Candida albicans A9540	12.5	12.5	3.13
Candida albicans ATCC 38247	12.5	12.5	3.13

to the corresponding trichloroethoxycarbonate **4** by treating with 2,2,2-trichloroethoxycarbonyl chloride (TrocCl) in CH_2Cl_2-pyridine. The coupling of **4** with 2,3,4,6-tetraacetyl-D-glucopyranose was conducted with diethyl azodicarboxylate (DEAD) and triphenylphosphine in THF to afford (+)-**5** with the desired β-configuration in 65% yield exclusively. The Mitsunobu reaction was also applicable to *N*-tosyl or *N*-ethoxycarbonyl analogs of **4**. Both acetyl and Troc groups of (+)-**5** were removed in methanolic NaOMe solution to furnish (+)-**1** in 44% yield. (−)-**1** was similarly synthesized from **4** and 2,3,4,6-tetraacetyl-L-glucopyranose. NMR spectra of both enantiomers of synthetic **1** were identical with those of natural **1**. The optical rotation values of synthetic **1** were +18.8° (c=0.1, pyridine) for D-glucoside and −14.7° (c=0.1, pyridine) for L-glucoside. We thus determined the absolute configuration of the sugar moiety of natural **1** was D-configuration since its $[\alpha]_D$ value was +16.5° (c=0.1, pyridine).

Biological Activities

The antimicrobial activities of both enantiomers of glucosylquestiomycin (**1**) and questiomycin A (**2**) are shown in Table 3. Questiomycin A inhibited the growth of both Gram-positive and -negative bacteria and yeasts with the IC_{50} value of 1.56~25 μg/ml. D-Glucosylquestiomycin showed a similar antibacterial spectrum to questiomycin A although it was less potent than questiomycin A. L-Glucosylquestiomycin did not show antibacterial activity against Gram-positive bacteria and *E. coli* but against *Pseudomonas* and yeasts. The cytotoxic activity of glucosylquestiomycin and questiomycin A was tested against U937 cells (Table 4). The IC_{50} value of D or L-glucoside (**1**) was 11~13 μg/ml whereas that of questiomycin A (**2**) was 0.15 μg/ml. These results indicate that the glycosylation of questiomycin A decreases its antibacterial and cytotoxic potency probably due to the low permeability through the cell membrane.

Table 4. Cytotoxity of D- and L-glucosylquestiomycin and questiomycin A against U937 cell line.

IC_{50} (μg/ml)		
D-Glucosyl-questiomycin	L-Glucosyl-questiomycin	Questiomycin A
11.2	12.5	0.15

Experimental

General

Melting points were determined on a Yanagimoto apparatus and are uncorrected. All NMR experiments were performed on a JEOL JNM-LA400 NMR spectrometer in the solvents specified. The MS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectra were recorded on a BECKMAN DU 640 spectrophotometer. IR spectra were recorded on a SHIMADZU FT IR-300 spectrophotometer. Optical rotations were measured on a HORIBA SEPA-300

polarimeter.

Acetylation of Glucosylquestiomycin

A solution of natural **1** (1.8 mg, 4.8 μmol) in a mixture of Ac_2O (100 μl) and pyridine (200 μl) was stirred for 18 hours at room temperature. Then the reaction mixture was poured into ice-water and extracted with EtOAc. The organic layer was washed with water, dilute aqueous HCl solution, saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was chromatographed on silica gel with the eluent of *n*-hexane - EtOAc (2:1) to give tetra-*O*-acetylglucosylquestiomycin **3** (2.3 mg, 88%).

2-(4',4',4'-Trichloroethoxycarbonylamino)-3*H*-phenoxazin-3-one (**4**)

To a solution of **2** (600 mg, 2.83 mmol) in a mixture of CH_2Cl_2 (12 ml) and pyridine (6 ml) was added 2,2,2-trichloroethyl chloroformate (1.2 ml, 8.49 mmol) at 0~5°C. After stirring for 12 hours at ambient temperature, the reaction mixture was poured into ice-water and extracted with EtOAc. The organic layer was washed with dilute aqueous HCl solution, saturated aqueous $NaHCO_3$ solution and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residual solid was crystallized from toluene - MeOH - acetone (2:1:1) to give **4** as a dark red powder (785 mg, 72%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.82 (2H, s, $-CH_2-CCl_3$), 6.46 (1H, s, 4-H), 7.39 (1H, dd, J=1.2 and 8.3 Hz, 6-H), 7.44 (1H, dt, J=1.2 and 7.8 Hz, 8-H), 7.57 (1H, s, 1-H), 7.63 (1H, ddd, J=1.5, 7.8 and 8.3 Hz, 7-H), 7.87 (1H, dd, J=1.5 and 8.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 75.96 (CH_2), 93.88 (CCl_3), 106.28 (4-C), 116.36 (6-C), 125.90 (8-C), 130.84 (9-C), 132.26 (1-C), 133.57 (9a-C), 133.74 (7-C), 139.09 (2-C), 143.84 (5a-C), 147.76 (10a-C), 149.33 (NH–CO), 149.70 (4a-C), 179.74 (3-C); IR (KBr) 1820, 1790, 1740, 1630, 1575, 1520, 1290, 1260, 1135 cm^{-1}; HRFAB-MS *m/z* 389.9700 $[M+H]^+$ (calcd *m/z* 386.9706 for $C_{15}H_{10}N_2O_4{}^{35}Cl_3$).

2-(*N*-4'',4'',4''-Trichloroethoxycarbonyl-2',3',4',6'-tetra-*O*-acetylglucopyranosylamino)-3*H*-phenoxazin-3-one (**5**)

(1) D-Glucosyl isomer

To a mixture of **4** (300 mg, 0.77 mmol), 2,3,4,6-tetra-acetyl-D-glucopyranose (0.67 g, 1.92 mmol) and Ph_3P (1.22 g, 4.65 mmol) in THF (50 ml) was dropwise added a 40% solution of diethyl azodicarboxylate in toluene (1 ml, 2.29 mmol) at 0~5°C. After stirring for 8 hours at room temperature, the reaction mixture was poured into ice-water and extracted with EtOAc. The organic layer was washed with water, saturated aqueous $NaHCO_3$ solution and brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was chromatographed on silica gel with the eluent of *n*-hexane - EtOAc (4:1 to 2:1) to give (+)-**5** (361 mg, 65%) as an orange powder: $[\alpha]_D^{27}$ +94.5° (*c* 0.6, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.82, 1.91, 1.94 and 2.19 (each 3H, each s, CH_3-CO-), 3.81 (1H, br. d, J=9.8 Hz, 5'-H), 4.16 (1H, dd, J=3.9 and 12.2 Hz, 6'-H), 4.22 (1H, br. d, J=12.2 Hz, 6'-H), 4.54 (1H, br. s, $-CH_2-CCl_3$), 4.71 (1H, br. d, J=11.0 Hz, $-CH_2-CCl_3$), 4.87 (1H, br. t, J=8.6 Hz, 2'-H), 5.00 (1H, t, J=9.8 Hz, 4'-H), 5.20 (1H, t, J=9.5 Hz, 3'-H), 5.76 (1H, br. d, J=9.3 Hz, 1'-H), 6.33 (1H, s, 4-H), 7.35 (1H, dd, J=1.2 and 8.3 Hz, 6-H), 7.41 (1H, dt, J=1.7 and 7.6 Hz, 8-H), 7.59 (1H, dt, J=1.2 and 7.8 Hz, 7-H), 7.72 (1H, s, 1-H), 7.81 (1H, dd, J=1.5 and 8.0 Hz, 9-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.24, 20.35, 20.45 and 20.55 (CH_3CO), 61.50 (6'-C), 66.96 (2'-C), 67.79 (4'-C), 73.69 (3'-C), 73.84 (5'-C), 75.92 (CH_2-CCl_3), 94.30 (CCl_3), 106.36 (4-C), 116.09 (6-C), 125.52 (8-C), 130.39 (9-C), 130.81 (1-C), 133.09 (7-C), 133.52 (9a-C), 139.03 (2-C), 143.72 (5a-C), 148.16 (10a-C), 149.19 (4a-C), 153.85 (NH–CO), 169.14, 169.53, 169.66 and 170.39 (CH_3CO), 180.14 (3-C); IR (KBr) 1755, 1640, 1580, 1520, 1230, 1040 cm^{-1}; HRFAB-MS *m/z* 718.0732 $[M+2H]^+$ (calcd *m/z* 718.0735 for $C_{29}H_{29}N_2O_{13}{}^{35}Cl_3$).

(2) L-Glucosyl isomer

In the same manner as described above, (−)-**5** was obtained from 2,3,4,6-tetraacetyl-L-glucopyranose in 61% yield: $[\alpha]_D^{27}$ −97.9° (*c* 0.62, $CHCl_3$); HRFAB-MS *m/z* 718.0732 $[M+2H]^+$ (calcd *m/z* 718.0735 for $C_{29}H_{29}N_2O_{13}{}^{35}Cl_3$).

2-Glucopyranosylamino-3*H*-phenoxazin-3-one (**1**)

(1) D-Glucosyl isomer

To a solution of (+)-**5** (0.27 g, 0.38 mmol) in MeOH (15 ml) was added a solution of 25% NaOMe in MeOH so that the pH was approximately 9 to indicator paper. After stirring for 18 hours at room temperature, the reaction mixure was neutralized by adding CH_3COOH and the resultant solution was adsorbed on a Diaion HP-20 column. The column was washed with water and eluted with acetone. The eluent containing the product was evaporated *in vacuo* and the residue was purified by ODS column chromatography with the eluent of CH_3CN - 0.15% KH_2PO_4 buffer (pH 3.5) (20:80) to

afford (+)-**1** (62 mg, 44%) as a red powder: $[\alpha]_D^{27}$ +18.8° (*c* 0.1, pyridine); NMR data were identical with those of natural **1** (Table 2); HRFAB-MS *m/z* 375.1182 $[M+H]^+$ (calcd *m/z* 375.1192 for $C_{18}H_{19}N_2O_7$).

(2) L-Glucosyl isomer

In the same manner as described above, (−)-**5** gave (−)-**1** in 51% yield: $[\alpha]_D^{28}$ −14.7° (*c* 0.1, pyridine); HRFAB-MS *m/z* 375.1174 $[M+H]^+$ (calcd *m/z* 375.1192 for $C_{18}H_{19}N_2O_7$).

Acknowledgments

We thank Drs. K. HARADA and K. FUJII at Meijo University for the measurement of mass spectra. This work was partly supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

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