Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 2229–2233

BIOORGANIC & MEDICINAL CHEMISTRY

Ptaquiloside Z, a New Toxic Unstable Sesquiterpene Glucoside from the Neotropical Bracken Fern *Pteridium Aquilinum* Var. *Caudatum*

Uvidelio Francisco Castillo,[a,*] Makoto Ojika,[a] Miguel Alonso-Amelot[b] and Youji Sakagami[a]

[a]*Department of Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*
[b]*Departamento de Química, Facultad de Ciencias, Universidad de Los Andes, Mérida 5101, Venezuela*

Received 29 June 1998; accepted 13 August 1998

Abstract—Reversed-phase HPLC fractionation, monitored by brine shrimp bioassay, led to the isolation of a new illudane-type sesquiterpene glucoside, ptaquiloside Z (**2**), as well as the known bracken carcinogen ptaquiloside (**1**), from a bioactive aqueous extract of the neotropical bracken fern *Pteridium aquilinum var. caudatum* (Pteridaceae). The structure of ptaquiloside Z (**2**) was confirmed by spectroscopic analyses and chemical degradation. Both compounds exhibited similar toxicity (LC_{50} 62.5 μg/ml at 24 h and LC_{50} 7.8 μg/ml at 48 h) toward brine shrimp.

Introduction

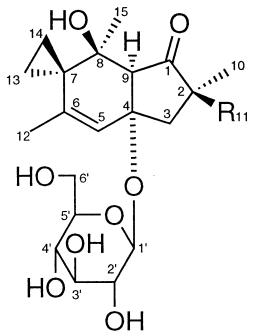
Pteridium aquilinum (L. Kuhn) (Pteridaceae) is considered to be one of the most successful and widely distributed organisms of the plant kingdom.[1,2] It has been recognized that the consumption of bracken fern by cattle induces bladder and intestinal carcinomas[3,4] and also causes a number of diseases in other farm animals.[5,6] Chemical investigation of more than 30 species of the Pteridaceae has been reported.[7] An active principle responsible for the carcinogenic and mutagenic activity was isolated simultaneously by Japanese[8,9] and Dutch[10] groups as an unstable norsesquiterpene glucoside named ptaquiloside (**1**) (aquilide A) (Fig. 1). Owing to the considerable economic importance of bracken as a farm animal hazard[11] and the discovery of the bracken carcinogen **1** in milk from bracken-fed cows,[12,13] we were stimulated to continue searching for other toxic components of this hazardous fern. To this end, we have conducted additional phytochemical studies of *P. aquilinum var. caudatum*,[14–16] one of the most commonly found neotropical varieties of bracken fern in the Northern Andes and Central America. The brine shrimp assay, which is known to be a convenient and short-term bioassay for toxic compounds, was used to test the isolated fractions.[17] We describe herein the isolation of a new ptaquiloside analogue, ptaquiloside Z (**2**), from the aqueous extraction of young fronds of *P. aquilinum* var. *caudatum*. The toxicity of **2** was the same as that of **1** in the brine shrimp assay.

Results and Discussion

Purification of a freeze-dried, hot-water extract of *P. aquilinum* var. *caudatum* croziers on ODS open column, using a water-to-acetonitrile gradient, yielded 19 fractions, from which fractions No. 5, 8, and 9 were observed to be the most toxic in the assay. Fractions No. 5, 8 and 9 were purified by reversed-phase preparative HPLC. Fractions No. 5 and 8 were found to contain known compounds, isoptaquiloside[13] and ptaquiloside (**1**),[8] respectively. Purification of fraction No. 9 by HPLC yielded two toxic components. One of the two was again identified by NMR analysis as ptaquiloside (**1**) (11.5 mg, $t_R = 51$ min). From ^1H and ^{13}C NMR spectral data (Table 1), the other component **2** (5.6 mg, $t_R = 72$ min) was presumed to be a new analogue of **1** and was named ptaquiloside Z. The obvious difference between the two compounds was that the secondary

*Corresponding author.

0968-0896/98/$—see front matter
PII: S0968-0896(98)00168-0



R = H: Ptaquiloside (**1**)
R = CH_3: Ptaquiloside Z (**2**)

Figure 1. Structures of ptaquiloside (**1**) and ptaquiloside Z (**2**).

methyl group of **1** was replaced with a geminal dimethyl fragment (δ 0.95, 1.08) in **2**. The molecular formula of **2** was determined to be $C_{21}H_{32}O_8$ by high-resolution FABMS and supported that **2** was a methylated analogue of **1**. Also, the following ^{1}H NMR signals similar to those of **1** were observed: two methyl signals (δ 1.20 and 1.49), one olefinic signal (δ 5.70), a cyclopropylidene signal (δ 0.53 to 0.82), an anomeric signal (δ 4.54), and a complex cluster of signals (δ 3.25 to 3.28). ^{13}C NMR data showed the presence of a ketone (δ 209.4) and an anomeric carbon signal (δ 98.2) as in the case of **1**. The assignment of the protonated carbons was performed by HMQC spectrum. The connectivity of the quaternary carbons and oxygen substituents was determined by HMBC data as shown in Figure 2. From these findings we were able to propose a gross structure of **2** (Fig. 2). The presence of the β-D-glucoside portion, though not evidenced by COSY and HMBC data, was unambiguously clarified by the following derivatization experiments. Acetylation of **2** yielded ptaquiloside Z tetraacetate (**3**). From the vicinal proton–proton coupling constants of the acetylated hexose moiety of **3**, we concluded that the hexose was glucose and it attached to the aglycon via a β-glycoside bond (Fig. 3). The tetraacetate **3** was then subjected to acidic methanolysis to yield two indanones, pterosin Z (**4**) and pterosin I (**5**), and methyl α- and β-D-glucosides, which were identified after acetylation and separation as methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside and its β-anomer. These results also confirmed the gross structure of **2**. Utilizing NOESY and ROESY NMR data, we were able to propose the relative stereochemistry of the aglycon part of **2**. The correlations of H-9/H-1′ and H-9/H-15 revealed that the ring A/B fusion was *cis*, and the β-glucosyl group, H-9 and H-15 adopted the α orientation (Fig. 4). The enantiotopic

Table 1. ^{13}C and ^{1}H NMR chemical shifts (δ, ppm) and coupling constants (Hz) determined for ptaquiloside Z (**2**) and its tetraacetate **3**.[a]

	Ptaquiloside Z (**2**)		Ptaquiloside Z tetraacetate (**3**)
Atom	^{13}C[b]	^{1}H[b]	^{1}H[c]
1	209.4 (s)		
2	46.4 (s)		
3α	51.1 (t)	2.15 d (13.4)	2.12 d (13.4)
3β		2.18 d (13.4)	2.16 d (13.4)
4	81.4 (s)		
5	124.0 (d)	5.70 s	5.68 (s)
6	142.5 (s)		
7	29.6 (s)		
8	71.2 (s)		
8-OH		4.48 s	4.26 s
9	61.9 (d)	2.75 s	2.65 s
10	26.6 (q)	1.08 s	1.06 s
11	25.7 (q)	0.95 s	0.95 s
12	19.0 (q)	1.49 s	1.51 s
13α	5.5 (t)	0.81 m	0.82 m
13β		0.66 ddd (9.6, 6.5, 4.1)	0.68 m
14α	9.4 (t)	0.53 ddd (8.9, 6.5, 4.1)	0.56 m
14β		0.84 m	0.86 m
15	26.6 (q)	1.20 s	1.08 s
1′	98.2 (d)	4.54 d (7.7)	4.83 d (8.1)
2′	74.5 (d)	3.08 dd (8.3, 7.7)	4.77 dd (9.4, 8.1)
3′	77.4 (d)	3.28 m	5.14 dd (9.6, 9.4)
4′	71.3 (d)[d]	3.25 m	4.87 dd (10.0, 9.6)
5′	76.5 (d)[d]	3.25 m	3.74 ddd (10.0, 6.3, 2.4)
6′	62.5 (t)	3.75 brd (11.3) 3.59 dd (11.3, 3.0)	3.98 dd (11.2, 6.3) 4.10 dd (11.2, 2.4)

[a] Measured in CD_3CN. Coupling constants are in parenthesis.
[b] Measured at 150 MHz for ^{13}C and at 600 MHz for 1 H.
[c] Measured at 400 MHz.
[d] Data interchangeable.

protons of the cyclopropane ring, H-3, and the geminal dimethyl groups were also assigned from the NOE correlations. The absolute stereochemistry of **2** was finally examined by applying the octant rule to ptaquiloside Z tetraacetate (**3**). We assumed that **3** possessed the same configuration as that of ptaquiloside tetraacetate (**6**), which was determined previously by X-ray crystalographic analysis.[18,19] The hydrogen atom at C-2 in the crystal structure of **6** was thus replaced with a methyl group and energy minimization with MM2 force field was performed to obtain a new molecular model of **3**

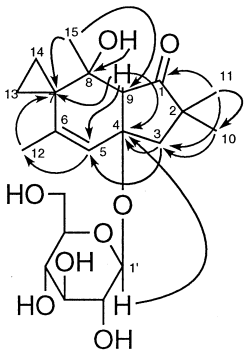


Figure 2. Gross structure from HMBC correlations (arrows) for ptaquiloside Z (**2**).

(Fig. 5). Since there were many more atoms in the minus areas of the back octants than in the plus areas, this model was expected to display a negative cotton effect. Actually, the CD spectrum of **3** (Fig. 6) showed a negative cotton effect at ca. 300 nm, which corresponds to the n−π* transition of the ketone group (C-1). The CD spectrum of ptaquiloside tetraacetate (**6**) also showed a negative cotton effect (Fig. 6). From these findings, we were able to conclude that the absolute stereochemistry of **2** was the same as that of **1**. Compound **2** showed toxicity as high as that of **1** after 24 and 48 h (LC_{50} 62.5 μg/ml and 7.8 μg/ml, respectively) against the brine shrimp *Arteima salina* LEACH.[17]

Experimental

Instruments

HPLC was performed on a JASCO high-pressure gradient system with PU-980 pumps and detection was carried out using a JASCO UV-970 at 220 nm. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded on a JASCO

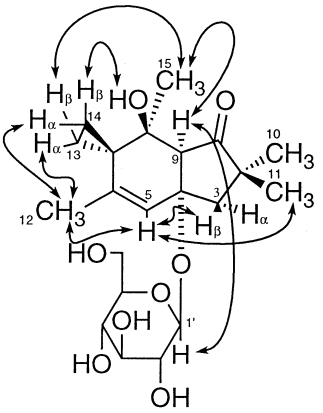


Figure 4. NOE correlations obtained from NOESY and ROESY for ptaquiloside Z (**2**).

Ubest-50 UV/VIS spectrometer. IR spectra were recorded on a JASCO FT/IR-7000S. Mass spectra were recorded on a JEOL Mstation JMS-700 mass spectrometer using sodium iodide/glycerol as a matrix in positive mode. One and two-dimensional (HMBC, HMQC, COSY, NOESY and ROESY) NMR spectra were obtained for CD_3CN and $CDCl_3$ solutions using a Bruker AMX-600 or a Bruker ARX-400 spectrometer. Chemical shifts are reported relative to TMS. ^{13}C NMR signal multiplicity was determined using DEPT 135 sequence. CD spectra were recorded on a JASCO J-720 spectropolarimeter. MM2 calculations in Chem3D ver. 3.5.1 were performed on an Apple Macintosh 7600/200 computer.

Plant material

Young fronds of bracken fern were collected between 8–12 days after emergence[14] at the site of "El Cerro La Bandera" in Mérida, Venezuela, during February 1996. They were identified using the key of Ortega[20] as *Pteridium aquilinum* var. *caudatum*. A voucher specimen (UVI 95-002) has been deposited in the herbarium of the

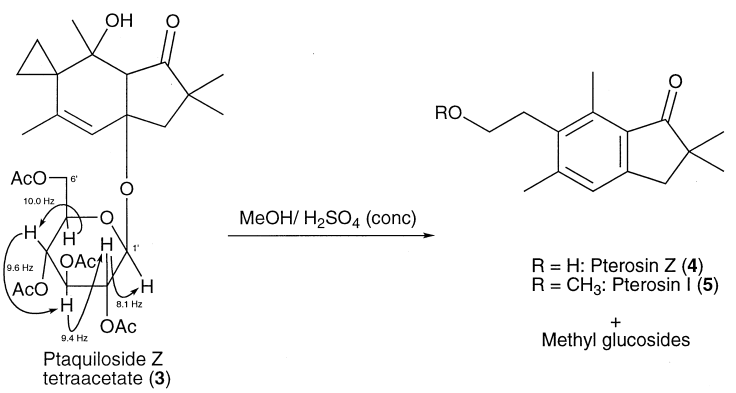


Figure 3. Ptaquiloside Z tetraacetate (**3**) degradation into two indanones **4** and **5** and methyl glucosides.

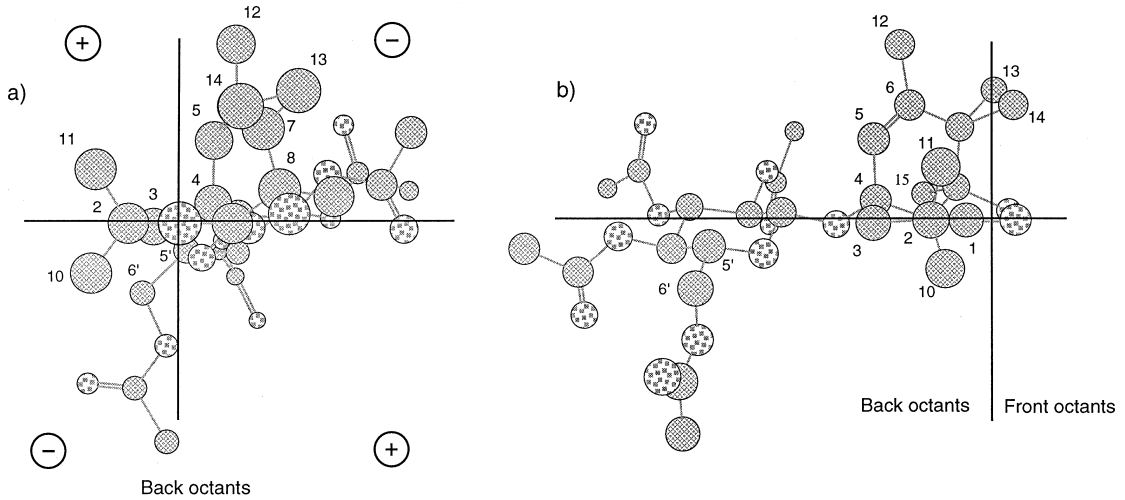


Figure 5. A plausible 3D conformation of **3** obtained from MM2 calculations. Projections were done on the plane of the keton group, viewed from the C = O axis (a) and from the side of the C = O group (b). The C-13 and C-14 carbons are located in a plus area of the front octants in (a).

Faculty of Pharmacy, Universidad de Los Andes, Mérida, Venezuela.

Extraction and purification procedures

Fresh fronds (1600 g) were mashed in hot boiling water using a high-power blender that reduced the plant tissue to an amorphous mass in which no fibers could be discerned under a mid-power scope. After blending, the mixture was cooled to 0 °C, filtrated and centrifuged. The supernatant was lyophilized to give a 58.4 g of solid material. This solid material was stored with silica gel at −30 °C. A portion of this material (20 g) was first fractionated by ODS (Cosmosil 75 C_{18}- OPN, 140 g, Nacalai Tesque) open-column chromatography and eluted using 100% H_2O to 100% CH_3CN to afford 19 fractions. Immediately, toxicity test was performed on each fraction. Fraction No. 9 (47.5 mg) was further purified by ODS preparative HPLC [column: Develosil ODS-HG-10 (10×250 nm), Nomura Chemical] using 37:63 MeOH:H_2O as an elution solvent. From this fractionation we obtained 2 compounds. The first compound (11.5 mg) (t_R = 51 min) was identified as ptaquiloside (**1**), $[\alpha]_D^{24}$ −184° (*c* 0.256, CH_3CN) [Lit. $[\alpha]_D^{24}$ −188° (*c* 1.00, MeOH)],[8] ^{1}H NMR (400 MHz, CD_3CN) δ 0.46 (ddd, *J* 9.5, 6.2, 4.0 Hz, 1H), 0.63 (ddd, *J* = 9.5, 5.8, 4.0 Hz, 1H), 0.76 (ddd, *J* = 9.5, 6.2, 4.1 Hz, 1H), 0.84 (ddd, *J* = 9.5, 5.8, 4.0 Hz, 1H), 1.04 (d, *J* = 6.9 Hz, 3H), 1.21 (s, 3H), 1.49 (d, *J* = 1.1 Hz, 3H), 1.87 (dd, *J* = 12.4, 12.4 Hz, 1H), 2.23 (m, 1H), 2.38 (dd, *J* = 12.4, 8.1 Hz, 1H), 2.54 (d, *J* = 1.3 Hz, 1H), 3.08 (dd, *J* = 7.9, 8.2 Hz, 1H), 3.26 (m, 3H), 3.58 (dd, *J* = 11.7, 5.0 Hz, 1H), 3.76 (dd, *J* = 11.7, 2.1 Hz, 1H), 4.39 (s, OH), 4.61 (d, *J* = 7.9 Hz, 1H), 5.68 (s, 1H). The second component (5.6 mg) (t_R = 72 min) was named ptaquiloside Z (**2**), $[\alpha]_D^{24}$ −168° (*c* 0.221, CH_3CN), IR (KBr) 3407 (br), 1717, 1645 cm^{-1}, UV (CH_3CN) λ_{max} 203 nm (ε 10500), HRFABMS *m/z* 435.2005 $[M+Na]^+$, calcd for $C_{21}H_{32}O_8Na$ 435.1982.

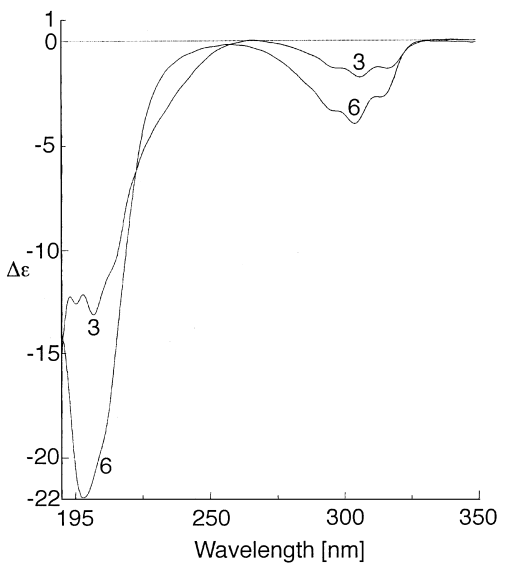


Figure 6. CD spectra of **3** and **6** in CH_3CN.

Ptaquiloside Z tetraacetate (3) and its degradation

Acetylation of **2** (2.06 mg) was done by standard acetylation procedure (Ac_2O, pyridine) to yield **3** (1.64 mg, 56.5%): CD (CH_3CN) λ_{ext} 316 (Δε −1.35), 313 (−1.28), 306 (−1.77), 298 (−1.33) nm. ^{1}H NMR data are shown in Table 1.

A mixture of **3** (1.64 mg) and concd. H_2SO_4 (10 µl) in MeOH (1 ml) was refluxed for 12 h, cooled to room temperature, neutralized with a solution of 1 M $NaHCO_3$ (160 µl) and evaporated under reduced pressure to give a solid. The solid was dissolved in water (3 ml) and extracted with $CHCl_3$ (10 ml). The $CHCl_3$ extract was concentrated to give an oily residue, which was separated by TLC on silica gel (6:1 CCl_4:acetone) to give two known compounds, pterosin Z (**4**) (0.18 mg, 11%) and pterosin I (**5**) (0.41 mg, 25%) which were readily identified by comparison of their reported NMR spectral data.[21,22] The aqueous portion was evaporated under reduced pressure to dryness, which was dissolved in a mixture of Ac_2O (0.5 ml) and pyridine (0.5 ml), and the mixture was stirred at room temperature for 5 h and concentrated. The resulting residue was dissolved in water (3 ml) and extracted with $CHCl_3$ (10 ml). The $CHCl_3$ extract was concentrated to give an oily residue, which was separated by TLC on silica gel (6:1 CCl_4:acetone) and identified by ^{1}H NMR and optical rotation as methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (0.37 mg, 23%), $[\alpha]_D^{24}$ +140° (*c* 0.019, $CHCl_3$) [Lit.$[\alpha]_D^{20}$ +130.5° ($CHCl_3$)],[23] and its β-anomer (0.09 mg, 5%).

Bioassay

All fractions were tested for toxicity utilizing brine shrimp (*Artemia salina* LEACH). The testing samples were made by serial dilution from 256 to 8 µg/ml in each well of a 24-well micro plate in 2 ml of artificial sea water. A suspension of nauplii containing 10 organisms (10 µl) was added to each well, and the covered plate was incubated at 25–27 °C for 48 h. Plates were observed every 24 h under a binocular microscope (×4) and the number of dead (non-motile) napulii in each well was counted. The 50% lethal concentrations (LC_{50}) at 24 and 48 h were measured by serial dilution media.

Acknowledgements

The authors are grateful to Professor Wolfgang Kraus (Universität Hohenheim) for providing us with information on the brine shrimp assay.

References

1. Taylor, J. A. In *Bracken Biology and Management*; (Eds.) Thomson, J. A.; Smith, R. T.; AIAS Occasional Publication, Australia, 1990; No. 40, pp 3–19.

2. Page, C. M. *Bot. J. Linn. Soc.* **1976**, *73*, 1–34.

3. Fenwick, G. R. *J. Sci. Food Agric.* **1995**, *46*, 147–173.

4. Smith B. L. In *Bracken Biology and Management*; (Eds.) Thomson, J. A.; Smith, R. T.; AIAS Occasional Publication, Australia, 1990; No. 40, pp 227–232.

5. Evans, W. C. In *Bracken Ecology, Land Used and Control Technology*; (Eds.) Smith, R. T.; Taylor, J. A.; Parthenon publishing, Carnforth, Lancashire, U.K; 1986, pp 121–132.

6. Evans, I. A. *Res. Vet. Sci.* **1979**, *26*, 339–348.

7. Murakami, T; Tanaka, N. *Prog. Chem. Org. Nat. Prod.* **1988**, *53*, 125–135.

8. Ojika, M.; Wakamatsu, K.; Niwa, H.; Yamada, K; Hirono, I. *Tetrahedron Lett.* **1983**, *24*, 4117–4120.

9. Ojika, M.; Wakamatsu, K.; Niwa, H.; Yamada, K. *Tetrahedron* **1987**, *43*, 5261–5274.

10. van der Hoeven, J. C. M.; Lagerweij, W. J.; Posthumus, M. A.; van Veldhuizen, A.; Holterman, H. A. *Carcinogenesis* **1983**, *4*, 1587–1592.

11. Alonso-Amelot, M.E.; Rodulfo-Baechler, S.; James-Espinoza, R. *Biochem. Syst. Ecol.* **1995**, *23*, 709–716.

12. Alonso-Amelot, M. E.; Castillo, U.; De Jongh, F. *Le Lait* **1992** *73*, 323–332.

13. Alonso-Amelot, M. E.; Castillo, U.; Smith, B. L.; Lauren, D. R. *Nature* **1996**, *382*, 587.

14. Alonso-Amelot, M. E.; Pérez-Mena, M.; Calcagno, M. P.; Jaimes-Espinoza, R.; Castillo, U. *J. Chem. Ecol.* **1992**, *18*, 1405–1420.

15. Castillo, U. F.; Wilkins, A. L.; Lauren, D. R.; Mc Naughton, D. E.; Smith, B. L. *J. Nat. Prod.* **1995**, *58*, 1889–1891.

16. Castillo, U. F.; Wilkins, A. L.; Lauren, D. R.; Smith, B. L.; Towers, N. R.; Alonso-Amelot, M. E.; Jaimes-Espinoza, R. *Phytochemistry* **1997**, *44*, 901–906.

17. Meyer, B. N.; Ferrigni, N. R.; Putman, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. *Planta Medica* **1982**, *45*, 31–34.

18. Niwa, H.; Ojika, M.; Wakamatsu, K.; Yamada, K.; Ohba, S.; Saito, Y.; Hirono, I.; Matsushita, K. *Tetrahedron Lett.* **1983**, *24*, 5371–5372.

19. Ohba, S.; Saito, Y.; Hirono, I.; Niwa, H.; Ojika, M.; Wakamatsu, K.; Yamada, K. *Acta Cryst.* **1984**, *C40*, 1877–1879.

20. Ortega, F. *Biollania* **1990**, *7*, 47.

21. Fukuoka, M.; Kuroyanagi, M.; Yoshihira, K.; Natori, S. *Chem. Pharm. Bull.* **1978**, *26*, 2365–2385.

22. Hayashi, Y.; Nishizawa, M.; Hirata, S.; Sakan, T. *Chem. Lett.* **1972**, 375–378.

23. *Dictionary of Organic Compounds*, 5th Edition; Buckingham, J. Ed.; Chapman and Hall, 1982; Vol. 4, 3866.