Bioorganic & Medicinal Chemistry Letters 20 (2010) 709–712

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel neofusapyrones isolated from *Verticillium dahliae* as potent antifungal substances

Miho Honma [a], Shinji Kudo [a], Noboru Takada [a], Kazuaki Tanaka [a], Tomisato Miura [b], Masaru Hashimoto [a,*]

[a] Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-Cho, Hirosaki, Aomori 036-8561, Japan
[b] Graduate School of Health Sciences, Hirosaki University, 66-1 Honcho, Hirosaki, Aomori 036-8564, Japan

ARTICLE INFO

Article history:
Received 25 September 2009
Revised 23 October 2009
Accepted 13 November 2009
Available online 3 December 2009

Keywords:
Isolation and structure determination
NMR spectral analysis
4-Hydroxy-2-pyrone
IR spectra
Theoretical ^{13}C NMR chemicalshift
Inhibition of MRSA clinical isolate 87-7927

ABSTRACT

Novel fusapyrone analogs, deoxyneofusapyrone and 7-desmethyldeoxyneofusapyrone were isolated from a pathogenic fungus, *Verticillium dahliae,* which causes Verticillium wilt disease in *Helianthus annuus*. Spectral analyses revealed that these are 2-pyrone type analogs of deoxyfusapyrone and its 7-desmethyl derivative, respectively. Biological assay disclosed that 10 μg of deoxyneofusapyrone inhibited the growth of MRSA clinical isolate 87-7927.

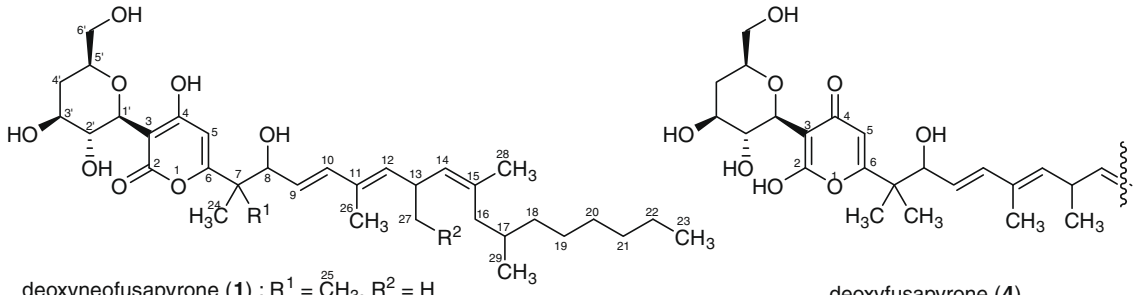
In the course of our investigation for novel biologically active compounds from fungi with unique ecologies, we have reported lambertellols[1–5] as mycoparasitic substances and cytotoxic spiroleptosphols.[6,7] Our continuous investigations led us to discover novel neofusapyrone derivatives, deoxyneofusapyrone (**1**) and 7-desmethyldeoxyneofusapyrone (**2**) from *Verticillium dahliae*, a pathogenic fungus that induces Verticillium wilt disease in *Helianthus annuus*.[8,9] The 2-pyrone structure in these compounds was established by comparison with IR spectral data in the literature as well as theoretical ^{13}C NMR chemical shifts. Biological assay disclosed that 10 μg of **1** inhibited the growth of MRSA clinical isolate 87-7927.

Agar disks containing cultured *V. dahliae* were found to powerfully inhibit growth of *Cochliobolus miyabeanus*[10] on potato-sucrose-agar (PSA) medium. The active substances were purified, based on observations of this activity, from 3.4 L of the cultured broth by successive chromatography with XAD-7, silica gel, ODS Sep-Pak®, and ODS HPLC to give deoxyneofusapyrone (**1**, 10.4 mg) and 7-desmethyldeoxyneofusapyrone (**2**, 6.9 mg), both as viscous oil.

Deoxyneofusapyrone (**1**, Fig. 1) showed a protonated molecular ion signal at m/z = 591.3925, which suggested a molecular formula of $C_{34}H_{54}O_8$ (the calculated mass for $[M+H]^+$ is 591.3900). The ^{13}C NMR spectrum afforded 34 resonance peaks, confirming the number of carbons in the molecule. DEPT spectra revealed seven methyl, eight methylene, twelve methyne, and seven quaternary carbons; thus suggesting 49 nonexchangeable protons in the molecule. Accordingly, **1** must possess five OH groups to satisfy the molecular formula. Detailed spectral analysis involving HMBC and NOESY, as shown in Table 1, disclosed that the present compound was a 2-pyrone isomer of known deoxyfusapyrone (**4**).[11] ^1H and ^{13}C NMR spectra of **1** were similar, but not identical to the data of **4** in the literature. Stereochemistries for the C9=C10, C11=C12, and C14=C15 double bonds were determined to be (*E*), (*E*), and (*Z*), respectively, by the coupling constants for C9H–C10H (J_{9H-10H} = 15.6 Hz), and the NOESY correlations for C8H↔C10H, C9H↔C26H_3, C10H↔C12H, C13H↔C16H, C13H↔C26H_3, and C14H↔C28H_3. The ^{13}C26 resonance signal was observed at a characteristically low frequency (13.03 ppm) also to support the *E*-configuration for the C11=C12 double bond because of steric compression with the bulky C13 group.[12] In addition, these experiments suggested a β-attached 2-deoxyglucopyranosyl group for the C1′–C6′ unit, which is an identical substructure to that of deoxyfusapyrone.[13] Chemical shift alteration in the ^{13}C NMR was remarkably localized around the pyrone ring between **1** and **4**. The ^{13}C chemical shifts for the pyrone ring moiety of **1** accorded more with those for neofusapyrone (**3**) than with those of **4,** as recently reported by Shiono.[14] Shiono proposed that Evidente's fusapyrone, originally proposed as a 4-hydroxy-2-pyrone derivative, should be revised to be 2-hydroxy-4-pyrone, and that their neofusapyrone (**3**) must possess a 4-hydroxy-2-pyrone ring on the basis

* Corresponding author.
E-mail address: hmasaru@cc.hirosaki-u.ac.jp (M. Hashimoto).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.11.063



deoxyneofusapyrone (**1**) : $R^1 = {}^{25}CH_3$, R^2 = H
7-desmethyldeoxyneofusapyrone (**2**) : R^1 = H, R^2 = H
neofusapyrone (**3**) : $R^1 = CH_3$, R^2 = OH

deoxyfusapyrone (**4**)

Figure 1.

Table 1
NMR spectral data for deoxyneofusapyrone (**1**) and 7-desmethyldeoxyneofusapyrone (**2**) in CD_3OD

No.	Deoxyneofusapyrone (**1**)				7-Desmethyldeoxyneofusapyrone (**2**)		
	^{13}C	^{1}H	HMBC	NOESY	^{13}C	^{1}H	HMBC
2	167.06	—			167.21	—	
3	100.30	—			100.48	—	
4	170.59	—			168.85	—	
5	100.86	6.08 (1H, s)	3, 4, 6, 7, 1′(*w*)	24, 25	102.27	6.05 (1H, s)	3, 4, 6, 7, 1′(*w*)
6	172.15	—			170.84	—	
7	45.52	—			46.56	2.61 (1H, dq, 7.0, 8.0)	4, 5, 8, 9, 24
8	78.16	4.32 (1H, dd, 1.1, 7.4)	6, 7, 9, 10, 24, 25	9, 10, 24, 25	75.88	4.21 (1H, t, 8.0)	4, 7, 9, 10, 24
9	125.97	5.55 (1H, dd, 7.4, 15.6)	8, 11	8, 24, 25, 26	127.66	5.51 (1H, dd, 8.0, 15.7)	7, 8, 11
10	139.20	6.23 (1H, d, 15.6)	8, 11, 12, 26	8, 12	139.04	6.23 (1H, d, 15.7)	8, 11, 12, 26,
11	131.46	—			131.87	—	
12	139.56	5.31 (1H, d, 9.2)	10, 13, 14, 26, 27	10, 27	139.75	5.34 (1H, d, 9.0)	10, 11, 13, 26, 27
13	32.99	3.42, (1H, m)	12, 14, 15, 27	16, 26, 27	32.99	3.42 (1H, m)	11, 12, 14, 15, 27
14	131.93	5.06 (1H, dd, 1.1, 9.4)	12, 13, 16, 27, 28	27, 28	131.38	5.06 (1H, dd, 1.0, 9.0)	16, 17, 27, 28
15	134.01	—			134.08	—	
16	40.99	1.95 (2H, m)	14, 15, 17, 18, 28, 29	13, 16	40.98	1.91 (2H, m)	14, 15, 17, 18, 28, 29
17	32.27	1.59 (1H, m)	26, 29	29	32.28	1.59 (1H, m)	16
18	38.31	1.10 (1H, m)	16, 29		38.33	1.13 (1H, m)	
		1.31(1H, m)				1.34 (1H, m)	
19	28.40	1.30 (2H, m)	18		28.39	1.28 (2H, m)	
20	30.71	1.28(2H, m)			30.71	1.28 (2H, m)	
21	33.07	1.28 (2H, m)			33.06	1.28 (2H, m)	
22	23.72	1.28 (2H, m)	23	23	23.72	1.28 (2H, m)	
23	14.47	0.89 (3H, t, 6.9)	21, 22	22	14.47	0.89 (3H, t, 6.8)	21, 22
24	20.39	1.21 (3H, s)	6, 7, 8, 25	5, 8, 9	15.32	1.13 (3H, d, 7.0)	6, 7, 8
25	23.00	1.15 (3H, s)	6, 7, 8, 24	5, 8, 9	—	—	
26	13.03	1.75 (3H, d, 1.1)	11, 12	9, 13	13.01	1.77 (3H, d, 1.0)	10, 11, 12
27	22.17	0.97 (3H, d, 6.6)	12, 13, 14	12, 13, 14	22.15	0.98 (3H, d, 6.8)	12, 13, 14
28	23.91	1.63 (3H, d, 1.1)	14, 15, 16, 27	14,	23.92	1.63 (3H, d, 1.0)	14, 15, 16
29	20.05	0.85 (3H, d, 6.5)	16, 17, 18	16, 17	20.04	0.85 (3H, d, 6.6)	15, 16, 17
1′	75.67	4.48 (1H, d, 9.5)	2, 3, 4, 2′, 5′	5′	75.70	4.48 (1H, d, 9.7)	2, 3, 6, 2′, 5′
2′	73.52	3.99 (1H, dd, 9.0, 9.5)	2, 1′, 3′	4′Hβ	73.44	4.00 (1H, dd, 9.2, 9.7)	2, 1′, 3′
3′	74.07	3.63 (1H, m)	2′, 4′		74.06	3.60 (1H, m)	
4′	36.58	1.57 (1H, m, 4′Hβ)	3′, 5′, 6′	2′	36.54	1.58 (1H, m)	2′, 5′, 6′
		1.95 (1H, m, 4′Hα)				1.95 (1H, m)	3′
5′	78.46	3.60 (1H, m)	6′, 4′, 1′	1′	78.44	3.60 (1H, m)	1′, 4′, 6′
6′	65.70	3.56 (2H, m)	4′, 5′		65.66	3.56 (2H, m)	4′, 5′

of empirical discussions of the C=O vibration signals in the IR spectra. They assigned a 2-pyrone structure for **3** by observing ν = 1675 cm^{-1} in the IR spectrum, because this wave number was close to those of previously reported 2-pyrones.[15,16] On the other hand, that for fusapyrone, revised to 4-pyrone by Shiono, was reported to be 1652 cm^{-1}.[14,17] The present sample showed ν = 1678 cm^{-1}, which closely accorded more with **3**. UV adsorption of **1** was observed at 288 nm, which also supported the same structure for the chromophore as that of **3** (λ_{max}, 289 nm). The wavelength for **4** (being revised to 4-pyrone) was 10 nm lower (λ_{max}, 280 nm) in the same solvent.[11]

However, the above discussion disagrees with the data in published textbooks.[18,19] It is mentioned that C=O adsorption of 2-pyrone appears at 1720–1740 cm^{-1}, while that for 4-pyrone is observed at 1650–1680 cm^{-1}. The wave number for the C=O adsorption of **1** (1678 cm^{-1}) suggests 4-pyrone structure rather than 2-pyrone when these tables are considered. To clarify this situation, we compared the theoretical ^{13}C NMR chemical shifts obtained by molecular orbital calculations with the experimental data.[2,20]

Model compounds **X** (2-pyrone) and **Y** (4-pyrone) were employed for the calculations. After structural optimization with HF 6-31G*, theoretical chemical shifts were calculated with the same basis set.[21] Although achiral models were chosen to minimize the number of conformers to be considered, conformers still existed around the hydroxyl group. Accordingly, the theoretical chemical

shifts were discussed after estimating their weighted average based on the Boltzmann distributions. The experimental shifts were plotted against the calculated shifts, and then the slope and intercept values were obtained by a linear least-squares fitting.[22] Employing these values, the calculated shifts were corrected to give the corrected ^{13}C chemical shifts. The difference $\Delta\delta$ values were obtained by subtracting the corrected ^{13}C shifts from the experimental data, yielding the chart shown in Figure 2. The experimental ^{13}C chemical shifts for **1** clearly showed better agreement with the theoretical shifts for model **X** than those for model **Y**. The average $|\delta\Delta|$ value for model **X** was 1.65 ppm, whereas that for model **Y** was 7.56 ppm. The root mean square (RMS) value between **1** and model **X** was 0.997, while that between model **Y** was lower (0.958). These results indicated that the chromophore part of **1** should be 4-hydroxy-2-pyrone, not 2-hydroxy-4-pyrone. Furthermore, because model **X** (2-pyrone) was 7.7 kcal/mol more stable than model **Y** (4-pyrone) in those calculations, the 2-pyrone form should abound even if tautomerization exists between them.[15] 4-Pyrone isomers have not so far been detected by our investigations of minor components by LC–MS.[23]

7-Desmethyldeoxyfusapyrone (**2**) was eluted slightly earlier than **1** in ODS HPLC. ESIMS (*m/z* = 577.3750) suggested a molecular formula of $C_{33}H_{52}O_8$, which is one CH_2 unit less than **1**. The ^{13}C NMR spectrum confirmed the number of carbons in the molecule. The ^1H NMR spectrum of **2** was quite similar to that of **1**, but lacked germinal methyl signals attached to C7. Doublet methyl (J = 7.0 Hz) and double-quartet methyne (J = 8.0, 7.0 Hz) signals were newly observed at 1.13 and 2.61 ppm, respectively. The methyne resonance due to C8H of **2** was observed as a triplet (J = 8.0 Hz), while that in **1** was a broad doublet (J = 7.4 Hz). Detailed analysis, including HMQC and HMBC spectra, disclosed that **2** was a 7-desmethyl derivative of **1** with the same stereochemistry, as shown in Figure 1. A similar desmethyl derivative but different side chain has been isolated as YM-202204 from *Phoma* sp. 60596.[24,25]

Finally, the biological properties of **1** and **2** were investigated. Both inhibited the growth of *C. miyabeanus*[10] in 100 ng/mL concentration. Assays employing clinical germs disclosed that 10 μg of **1** induced a clear inhibition circle against MRSA clinical isolate 87-7927 on a soybean–casein digest agar medium (Difco).[26] Because neither of them showed cytotoxicity against HeLa (human cervix carcinoma) and HepG2 (human hepatocellular carcinoma), **1** might have potential as a safe anti-MRSA agent.

As described, we disclosed deoxyneofusapyrone (**1**) and 7-desmethyldeoxyneofusapyrone (**2**) from *V. dahliae*. Notably, these compounds were produced by an onshore fungus isolated from *H. annuus*, although other fusapyrone derivatives, and the related YM-202204[24] were isolated from marine fungi. Further biological properties of these compounds are now under investigation in our laboratories.

Isolation and physical data for 1 and 2: V. dahliae isolated from *H. annuus* at Aomori prefecture in Japan in 1988 was cultured in potato (680 g)-sucrose (68 g) media (200 mL × 17) employing baffled flasks at 25 °C for 23 days under stirring conditions (130 rpm). Each culture medium was diluted with MeOH (200 mL), combined, and filtered through a Celite® pad. The filtrate was concentrated by a rotary evaporator below 35 °C until the whole volume became ca. 1.0 L. The resulting aqueous solution was loaded on an XAD-7 (800 g). After washing successively with 20% and 40% MeOH/H_2O, the fractions eluted with 60% MeOH/H_2O were collected and concentrated to give a crude solid (300 mg), which was further fractionized by silica gel column chromatography (50% MeOH/AcOEt) and ODS Sep-Pak® (10 g, 90% MeOH/H_2O), yielding a crude sample. HPLC purification (Waters μ-bondasphere C18, 150 × 19 mm I.D. 70:30 CH_3CN/H_2O containing 0.1% TFA, 10 mL/min flow) yielded **1** (10.4 mg, t_R = 24 min) and **2** (6.9 mg, t_R = 20 min), both as viscous oil.

Deoxy-neofusapyrone (**1**): $[\alpha]_D^{25}$ −16 (*c* 0.23, MeOH), UV (8.4 × 10^{-5} mol/L in MeOH) λ_{max} 205 (ε 17,000), 237 (ε 12,000), 288 (ε 5000) nm, IR (neat) 3379, 2924, 1678, 1385 cm^{-1}.

7-Desmethyldeoxyneofusapyrone (**2**): $[\alpha]_D^{25}$ −29 (*c* 0.38, MeOH), UV (8.6 × 10^{-5} mol/L in MeOH) λ_{max} 205 (ε 15,000), 238 (ε 16,000), 288 (ε 5000) nm, IR (neat), 3382, 2927, 1682, 1435 cm^{-1}.

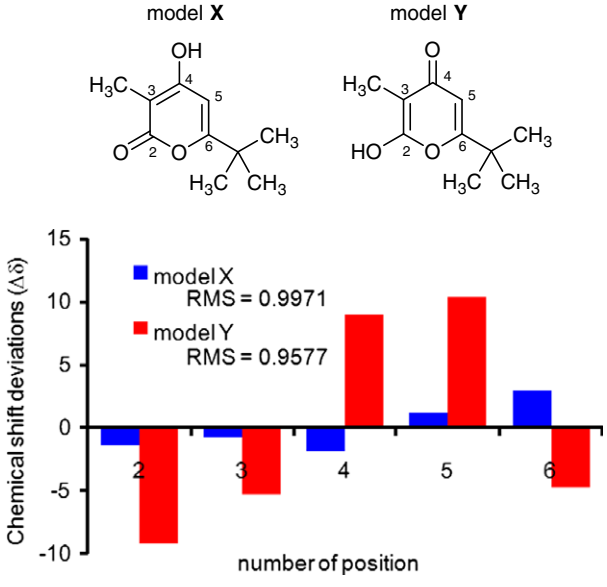


Figure 2. Structures of models **X** and **Y**, and $|\delta\Delta|$ values between experimental and calculated ^{13}C chemical shifts.

Acknowledgments

This study was partly supported by Grant-in-Aid for Scientific Research (No. 21390069) from the Japan Society for Promotion of Science. We are grateful to Professor Yoshihito Shiono of Yamagata University and Dr. Yoko Saikawa of Keio University for fruitful discussion.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.11.063.

References and notes

1. Schuetz, A.; Murakami, T.; Takada, N.; Junker, J.; Hashimoto, M.; Griesinger, C. *Angew. Chem., Int. Ed.* **2008**, *47*, 2032.
2. Murakami, T.; Takada, N.; Hehre, W. J.; Hashimoto, M. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4228.
3. Murakami, T.; Sasaki, A.; Fukushi, E.; Kawabata, J.; Hashimoto, M.; Okuno, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2587.
4. Murakami, T.; Hashimoto, M.; Okuno, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4185.
5. Murakami, T.; Morikawa, Y.; Hashimoto, M.; Okuno, T.; Harada, Y. *Org. Lett.* **2004**, *6*, 157.
6. Murakami, T.; Tsushima, T.; Takada, N.; Tanaka, K.; Nihei, K.; Miura, T.; Hashimoto, M. *Bioorg. Med. Chem.* **2009**, *17*, 492.
7. Hashimoto, M.; Tsushima, T.; Murakami, T.; Nomiya, M.; Takada, N.; Tanaka, K. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4228.
8. Robert, R. G.; Robertson, J. A.; Hanlin, R. T. *Can. J. Bot.* **1986**, *64*, 1964.
9. Fradin, E. F.; Thomma, B. P. H. *Mol. Plant Pathol.* **2006**, *7*, 71.
10. Nihei, K.; Itoh, H.; Hashimoto, K.; Miyairi, K.; Okuno, T. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 852.
11. Evidente, A.; Conti, L.; Altomare, C.; Bottalico, A.; Sindona, G.; Segre, A. L.; Logrieco, A. *Nat. Toxins* **1994**, *2*, 4.
12. Dalling, D. K.; Grant, D. M. *J. Am. Chem. Soc.* **1972**, *94*, 5318.
13. The absolute chemistry was not studied in these studies.
14. Hiramatsu, F.; Miyajima, T.; Murayama, T.; Takahashi, K.; Koseki, T.; Shiono, Y. *J. Antibiot.* **2006**, *59*, 704.

15. Xaio, J.-Z.; Kumazawa, S.; Yoshikawa, N.; Mikawa, T.; Sato, Y. *J. Antibiot.* **1993**, *46*, 48.
16. Hussain, R. A.; Waterman, P. G. *Phytochemistry* **1982**, *21*, 1393.
17. Evidente reported that 1725 cm^{-1} by measuring the sample after saponification of the corresponding acetate. They also reported 1647 cm^{-1} but assigned as adsorption by triene substructure.
18. Nakanishi, K.; Solomon, P. H. *Infrared Adsorption Spectroscopy*, 2nd ed.; Emerson-Adams Press: Boca Raton, Florida, 1998 (see Ref. 11).
19. Pretsch, E.; Buhlmann, P.; Affolter, C. *Structure Determination of Organic Compounds: Tables of Spectral Data*; Springer: Berlin, 2000.
20. Rychnovsky, S. D. *Org. Lett.* **2006**, *8*, 2895.
21. SPARTAN *06 Version1.1.2* (Wavefunction Inc., 18401 Von Karman Avenue, Suite 370, Irvine, CA 92612, USA) was used for these calculations.
22. Model **X** versus **1**; slope 0.955, intercept 15.768, model **Y** versus **1**; slope 0.974, intercept 7.560.
23. The NMR data for Evidente's fusapyrone (see Ref. 11) matched more with model **Y** (RMS = 0.986) than model **X** (RMS = 0.967) by similar calculations, which may support the 4-pyrone structure in the case of their compound.
24. Nagai, K.; Kamigiri, K.; Matsumoto, H.; Kawano, Y.; Yamaoka, M.; Shimoi, H.; Watanabe, M.; Suzuki, K. *J. Antibiot.* **2002**, *55*, 1036.
25. It was difficult to discuss whether YM-202204 was a 2-pyrone or 4-pyrone form because the IR adsorption for this compound was reported to be 1665 cm^{-1}.
26. We determined antimicrobial activity also against *Escherichia coli* NIHJ Jc-2, *Staphylococcus aureus* ATCC25923, and *Pseudomonas aeruginosa* ATCC27853. However, neither **1** nor **2** induced remarkable inhibition of those.