NOVEL ANTINEMATODAL AND ANTIPARASITIC AGENTS FROM *PENICILLIUM CHARLESII*

II. STRUCTURE DETERMINATION OF PARAHERQUAMIDES B, C, D, E, F, AND G

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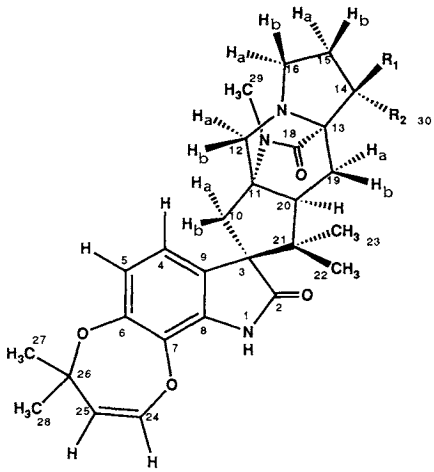
Paraherquamides B (**2**, $C_{27}H_{33}N_3O_4$), C (**3**, $C_{28}H_{33}N_3O_4$), D (**4**, $C_{28}H_{33}N_3O_5$), E (**5**, $C_{28}H_{35}N_3O_4$), F (**6**, $C_{28}H_{35}N_3O_3$), and G (**7**, $C_{28}H_{35}N_3O_4$) are novel metabolites of *Penicillium charlesii*. The structures of these compounds have been determined by NMR and MS analysis.

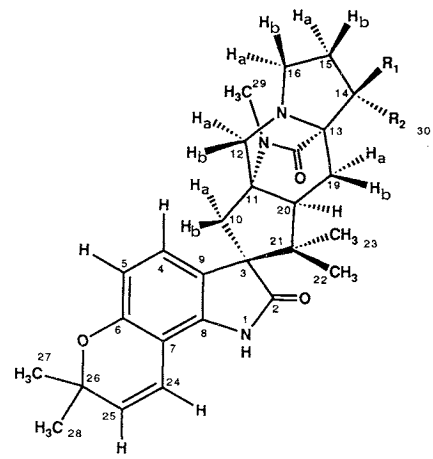
The fermentation, isolation, and characterization of six novel analogs (**2**~**7**) of paraherquamide[1] (**1**) have been reported in the preceding paper[2]. In this paper, the structure determination of these compounds is described.

Results and Discussion

The structures of the present compounds were determined based upon interpretation of their NMR and MS and comparison with previously reported paraherquamide[1] (**1**). Paraherquamide (**1**) has the MW 493 ($C_{28}H_{35}N_3O_5$) and exhibits critical fragment ions at *m/z* 434, 165, and 163 in its EI-MS. The fragment ion at *m/z* 434 corresponds to neutral loss of *N*-methylformamide from the molecular ion. Cleavage of bonds C-20~C-21 and C-10~C-3 (see **1**) with loss of *N*-methylformamide affords *m/z* 163, the saturated



1 R_1=OH R_2=CH_3
2 R_1=H R_2=H
3 R_1, R_2=H_2C=
4 R_1, R_2=$-OCH_2-$
5 R_1=H R_2=CH_3



6 R_1=H R_2=CH_3
7 R_1=OH R_2=CH_3

Table 1. ^{1}H NMR data for paraherquamides (**1**~**7**)[a].

Proton	**1**[b]	**1**	**2**	**3**
4-H	7.03 d (8.1)	6.84 d (7.3)	6.85 d (8.2)	6.86 d (8.2)
5-H	6.66 d (8.1)	6.68 d (7.3)	6.67 d (8.2)	6.68 d (8.0)
10-Ha	2.67 d (15.3)	2.65 d (16.1)	2.67 d (15.2)	2.68 d (15.2)
10-Hb	1.97 d (15.4)	1.87 d (15.6)	1.87 d (15.1)	1.88 d (15.4)
12-Ha	2.58 dd (1.8, 11.4)	2.55 dd (2.2, 11.0)	2.61 dd (1.0, 11.5)	2.68 dd (1.2, 11.2)
12-Hb	3.65 d (11.4)	3.58 d (10.8)	3.59 d (10.9)	3.63 d (10.7)
15-Ha	1.75[c] dd	1.80[c] m	1.39[c] m	2.55[c] m
15-Hb	2.18[c] m	2.24[c] m	2.13[c] m	2.55[c] m
16-Ha	2.14[c] m	2.18[c] m	2.13 m	2.29[c] m
16-Hb	3.15[c] ddd	3.17[c] ddd	3.03 m	3.06[c] m
19-Ha	1.83 dd (10.3, 12.9)	1.77 dd (10.8, 10.8)	1.83 m	2.03 dd (11.4, 12.3)
19-Hb	1.71 dd (11.0, 12.9)	1.75 dd (10.5, 12.5)	1.64 dd (10.4, 12.4)	1.65 dd (9.5, 12.3)
20-H	3.00 ddd (1.7, 10.5, 10.5)	2.96 ddd (2.0, 10.3, 10.3)	2.98 m	3.06 m
22-H	1.11 s	1.08 s	1.09 s	1.09 s
23-H	0.82 s	0.84 s	0.83 s	0.86 s
24-H	6.36 d (7.7)	6.32 d (7.8)	6.32 d (7.7)	6.33 d (7.7)
25-H	4.97 d (7.8)	4.90 d (6.8)	4.89 d (7.7)	4.90 d (7.7)
27-H	1.39[d] s	1.41[d] s	1.41[d] s	1.41[d] s
28-H	1.42[d] s	1.43[d] s	1.43[d] s	1.44[d] s
29-H	2.99 s	2.99 s	3.01 s	3.01 s
14-OH	2.75 br s	2.66 br s	—	—
1-H	9.45 br s	7.50 br s	7.41 br s	7.41 br s
14-H	—	—	1.83[d] m, 2.49[d] m	—
30-H	1.54 s	1.56 s	—	4.97 m (<2), 5.13 m (<2)

Proton	**4**	**5**	**6**	**7**
4-H	6.86 d (8.2)	6.84 d (8.5)	6.94 d (8.4)	6.93 d (8.5)
5-H	6.68 d (8.2)	6.67 d (7.5)	6.42 d (8.3)	6.42 d (8.2)
10-Ha	2.66 d (15.4)	2.66 d (15.9)	2.64 d (15.0)	2.64 d (15.6)
10-Hb	1.89 d (15.4)	1.86 d (16.0)	1.83 d (15.8)	1.85 d (15.7)
12-Ha	2.61 dd (1.4, 11.2)	2.51 br d (10.6)	2.50 dd (1.0, 10.7)	2.56 dd (1.5, 11.5)
12-Hb	3.67 d (10.9)	3.56 br d (10.7)	3.54 d (11.4)	3.58 br d (11.2)
15-Ha	2.36 ddd (1.5, 8.8, 13.6)	1.75 dddd (4.5, 10.7, 10.7, 10.7)	1.75 dddd (4.5, 10.6, 10.6, 10.6)	1.79 m
15-Hb	1.92 ddd (8.9, 8.9, 13.7)	1.97 m	1.97 m	2.25 m
16-Ha	2.27 ddd (8.6, 8.6, 8.6)	2.20 m	2.20 ddd (5.5, 8.9, 10.6)	2.20 m
16-Hb	3.22 ddd (1.5, 9.0, 9.0)	3.13 m	3.12 ddd (4.4, 9.0, 9.0)	3.18 ddd (4.5, 9.0, 9.0)
19-Ha	1.57 dd (9.9, 12.4)	2.00 dd (11.0, 11.2)	2.00 dd (11.1, 11.5)	1.82 dd (10.6, 12.8)
19-Hb	1.41 m	1.38 m	1.38 m	1.76 dd (10.4, 12.9)
20-H	2.96 m	2.96 m	2.98 m	2.98 ddd (1.5, 10.8, 10.8)
22-H	1.10 s	1.08 s	1.07 s	1.09 s
23-H	0.82 s	0.84 s	0.83 s	0.84 s
24-H	6.32 d (7.7)	6.33 d (8.2)	6.34 d (9.6)	6.40 d (9.8)
25-H	4.90 d (7.7)	4.90 d (8.0)	5.77 d (9.7)	5.73 d (9.9)
27-H	1.41[d] s	1.41[d] s	1.41[d] s	1.41[d] s
28-H	1.43[d] s	1.43[d] s	1.44[d] s	1.44[d] s
29-H	3.01 s	2.97 s	2.97 s	3.00 s
14-OH	—	—	—	2.69 br s
1-H	7.41 br s	7.57 br s	8.32 br s	8.96 br s
14-H	—	1.89 m	1.89 m	—
30-H	2.93 d (4.3), 3.08 d (4.3)	1.36 d (6.8)	1.37 d (6.9)	1.57 s

[a] Spectra recorded at 400 MHz in CD_2Cl_2 except where noted. Chemical shifts in ppm referenced to CD_2Cl_2 at 5.32 ppm as internal standard. Data in parentheses are coupling constants in J=Hz. ddd=doublet of doublet of doublets. dddd=doublet of doublet of doublet of doublets.

[b] Spectrum taken in acetone-d_6, referenced to solvent peak at 2.04 ppm as internal standard.

[c] Spin system showing second order effects, observed coupling constants are not accurate.

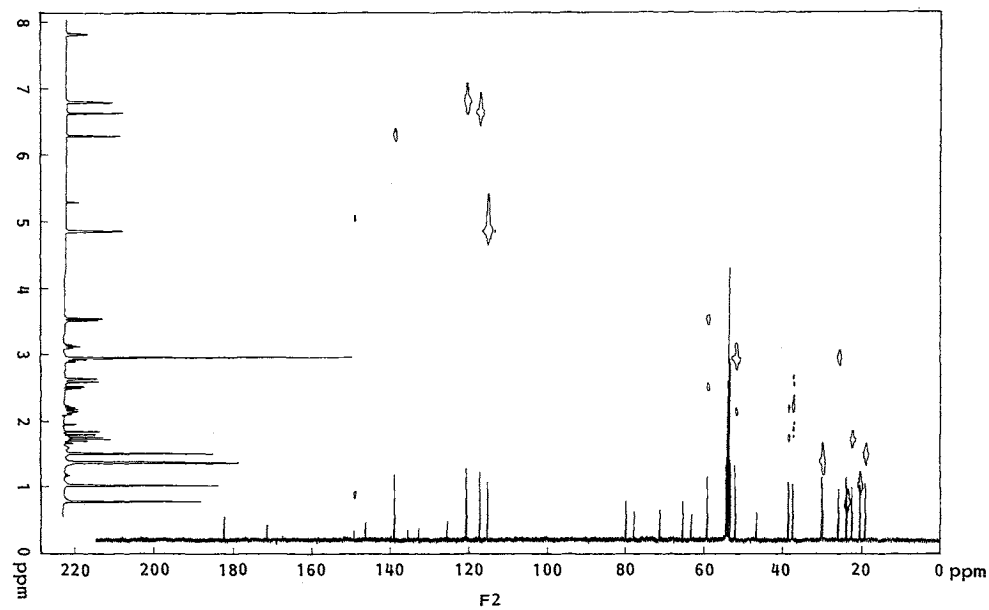
[d] Interchangeable assignments.

Table 2. ^{13}C NMR data for **1**.

Carbon	δ (ppm)	m	$^1J_{C-H}$	Carbon	δ (ppm)	m	$^1J_{C-H}$
C-2	182.34	s	—	C-16	52.04	t	140
C-3	63.32	s	—	C-18	171.47	s	—
C-4	120.73	d	161	C-19	22.53	t	133
C-5	117.31	d	162	C-20	51.97	d	130
C-6	146.23	s	—	C-21	46.62	s	—
C-7	135.48	s	—	C-22	20.59	q	126
C-8	132.71	s	—	C-23	23.91	q	126
C-9	125.48	s	—	C-24	139.06	d	193
C-10	37.44	t	131	C-25	115.30	d	155
C-11	65.55	s	—	C-26	80.00	s	—
C-12	59.27	t	135	C-27	29.92	q	127
C-13	71.46	s	—	C-28	30.06	q	128
C-14	78.03	s	—	C-29	25.96	q	129
C-15	38.57	t	131	C-30	19.17	q	138

Chemical shifts in ppm referenced to CD_2Cl_2 at 53.8 ppm as internal standard. All coupling constants are in J=Hz. Carbon numbering is based on that of the marcfortines[3].
m: Multiplicity.

Fig. 1. One-bond ^{13}C-^1H chemical shift correlation (HETCOR) NMR spectrum for paraherquamide (**1**) at 19°C in CD_2Cl_2 at 100 MHz.



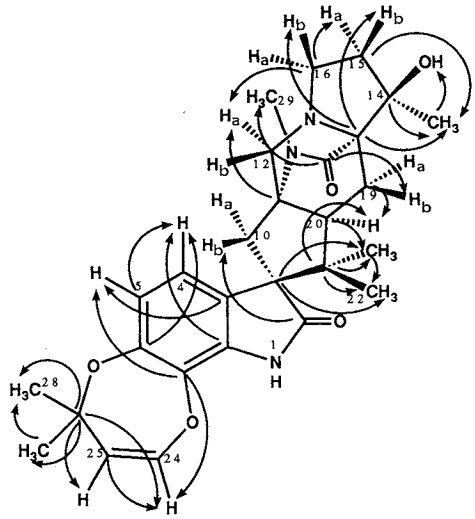
fused ring portion of the molecule. The lower intensity *m/z* 165 ion appears to result from loss of methylisocyanate ($CH_3N{=}C{=}O$) rather than *N*-methylformamide.

The published ^1H NMR spectrum[1] of **1** in $CDCl_3$ is complicated by the poor spectral characteristics in this solvent. Subsequent ^{13}C and ^1H NMR studies with **1** in CD_2Cl_2 and $(CH_3)_2CO$-d_6 led to complete assignment of all the resonances (Tables 1 and 2). In particular, ^{13}C-^1H correlation experiments (HETCOR, Fig. 1) were critical in definitively assigning the following proton resonances on the

terminal pyrrolidine ring: 3.17 and 2.18 ppm to C-16 (52.04 ppm), and 2.24 and 1.80 ppm to C-15 (38.57 ppm). Two and three bond ^{13}C-^{1}H correlations (see Fig. 2) determined by a long-range HETCOR experiment and long-range coupling data from the 'gated' ^{13}C spectrum confirmed the ^{13}C assignments. These assignments include several reassignments of the carbon resonances for the marcfortines as previously published[3].

Fig. 2. Multiple bond ^{13}C-^{1}H chemical shift correlation data obtained from HETCOR experiments[8] for paraherquamide (**1**) in CD_2Cl_2.

Two- and three-bond carbon-hydrogen shift correlations are shown with arrows.



The molecular formula $C_{27}H_{33}N_3O_4$ was determined for paraherquamide B (**2**) by HR-MS. This formula is CH_2O less than that of **1**. A strong $(M-59)^+$ ion is observed at *m/z* 404 which suggests the presence of the paraherquamide *N*-methylamide moiety. The critical ions at *m/z* 133 and 135 reflect the CH_2O difference (30 amu, *cf.* 163 and 165) and indicate that the change is in the saturated fused ring system, presumably at C-14. Consideration of the ^{1}H NMR data (Table 1), including COSY data, led to the C-14-desmethyl, C-14-deshydroxy structure **2**. The methyl singlet resonance in the proton spectrum at 3.01 ppm indicated the presence of the amide *N*-methyl functionality. The absence of the C-14-methyl and the C-14-hydroxyl resonances observed at 1.56 and 2.66 ppm, respectively, in **1** and the addition of two new one proton multiplets at 1.83 and 2.49 ppm (14-H) confirmed the absence of the methyl and hydroxyl substituents on the pyrrolidine ring.

Paraherquamide C (**3**) has the molecular formula $C_{28}H_{33}N_3O_4$ by HR-MS, and this formula is H_2O less than that of **1**. A strong $(M-59)^+$ ion is observed at *m/z* 416 and the critical saturated fused ring ions are observed at *m/z* 145 and 147 rather than at *m/z* 163 and 165 as in **1**, thus reflecting the absence of the equivalent of H_2O presumably at the C-14 position. ^{1}H NMR data (Table 1), including COSY, showed an absence of the C-14-methyl and C-14-hydroxyl proton resonances observed in **1** and their replacement with two one proton resonances at 4.97 and 5.13 ppm (30-H). The four spin system involving the methylene protons on carbons 15 and 16 remained, with the two resonances on C-15 downfield shifted to 2.55 ppm from 1.80 and 2.24 ppm as in **1**. This data, supported by the small coupling constants of the 4.97 and 5.13 ppm (30-H) olefinic resonances (see Table 1), led to the assignment of the exocyclic vinylic moiety involving C-14 and C-30 and structure **3** for paraherquamide C.

The molecular formula $C_{28}H_{33}N_3O_5$ was determined for paraherquamide D (**4**) by HR-MS and this formula is H_2 less than that for **1**. The additional unsaturation in **4** is generally located in the saturated fused ring portion of the molecule by the critical ions at *m/z* 161 and 163 which are down 2 mass units form 163 and 165 as in **1**. An intense $(M-59)^+$ is again observed at *m/z* 432 indicating the presence of the standard *N*-methylamide moiety. ^{1}H NMR data showed the presence of the amide *N*-methyl singlet at 3.01 ppm. The C-14-hydroxyl and C-14-methyl proton resonances were absent and two doublets were observed at 2.93 and 3.08 ppm (30-H) each with a coupling constnat of $J=4.3$ Hz. ^{1}H COSY indicated that these resonances were coupled to each other, and the chemical shifts and coupling constants of these

resonances together with the absence of the C-14-methyl resonance observed for **1** suggested the presence of a spiro-epoxide moiety. The location of the epoxide on the pyrrolidine ring was confirmed by analysis of the remaining resonances. A four spin system consisting of resonances at 1.92 (15-Hb), 2.36 (15-Ha), 2.27 (16-Ha) and 3.22 (16-Hb) ppm was assigned to the methylene protons on carbons 15 and 16. The relative stereochemistry of the protons was assigned on consideration of the coupling constants and ring geometry. The $J=1.5$ Hz coupling between the 3.22 (16-Hb) and 2.36 (15-Ha) ppm resonances implied a near 90 degree dihedral angle between these protons. This established a $J=9$ Hz geminal coupling between the 3.22 (16-Hb) and 2.27 ppm (16-Ha) resonances and a $J=13.7$ Hz geminal coupling between the 2.36 (15-Ha) and 1.92 ppm (15-Hb) resonances. Both these values are in good agreement with observed literature geminal coupling constants for pyrrolidine ring systems[4]. ^{13}C NMR data including Attached proton test[5], used to establish carbon multiplicities confirmed the structural assignment. In comparison with paraherquamide, the carbon data indicated an upfield shift for C-15 from 38.57 to 31.09 ppm and for C-14 from 78.03 to 63.00 ppm, and replacement of the C-30 (19.17 ppm) methyl signal with a methylene resonance at 46.48 ppm.

Paraherquamide E (**5**) has the molecular formula $C_{28}H_{35}N_3O_4$ by HR-MS and this formula is O (oxygen) less than that of **1**. A strong fragment ion at *m/z* 418 which corresponds to $(M-59)^+$ is observed suggesting the presence of the standard *N*-methylamide moiety. The critical ions at *m/z* 147 and 149 reflect the 16 mass unit difference from **1** and indicate that the oxygen is missing from the saturated fused ring portion of the molecule, presumably from C-14. The ^1H NMR spectrum obtained for **5** was quite similar to that of **1**. The critical differences noted were the absence of the C-14-hydroxyl resonance, the appearance of a new methine multiplet at 1.89 ppm (14-H), and the change in the C-14-methyl resonance from a singlet at 1.56 ppm to an upfield shifted doublet ($J_{H-H}=6.8$ Hz) at 1.36 ppm. These observations allowed structural assignment of **5** as the C-14-deshydroxy derivative of **1**.

The molecular formula $C_{28}H_{35}N_3O_3$ was determined for paraherquamide F (**6**) by HR-MS and this formula is O_2 less than that of **1**. The standard *N*-methylamide moiety is indicated by the strong $(M-59)^+$ at *m/z* 402. The critical ions at *m/z* 147 and 149 indicate that one oxygen is missing from the saturated fused ring portion of the molecule, presumably from C-14, and by difference, the second oxygen equivalent must be missing from the indolone portion of the molecule. The ^1H NMR spectrum showed the absence of the C-14-hydroxyl resonance and the change in the C-14-methyl resonance (1.56 ppm singlet to 1.37 ppm doublet coupled to 1.89 ppm (14-H) methine multiplet) as observed for **5**. In addition, the spectrum of **6** exhibited a downfield shift of the C-25 olefinic proton resonance from 4.90 to 5.77 ppm with a change in the 24-H~25-H coupling constant from $J=7.8$ to 9.7 Hz. These changes are consistent with deletion of the oxygen α to the C-24~C-25 double bond to form a 2,2-dimethyl-α-pyran ring as in **6** rather than the dihydro-1,4-dioxepin of **1**.

Paraherquamide G has the molecular formula $C_{28}H_{35}N_3O_4$ by HR-MS and this formula is O (oxygen) less than that of **1**. The characteristic $(M-59)^+$ ion suggests the standard *N*-methylamide moiety. The critical ions observed at *m/z* 163 and 165 as in **1**, suggest by difference that the oxygen must be missing from the indolone portion of the molecule. The ^1H NMR data for **7** displayed a close similarity to **1** except for the C-25 olefinic proton which was shifted downfield to 5.73 ppm with a concomitant change in the 24-H~25-H coupling constant as observed in **6**, again indicating a 2,2-dimethyl-α-pyran ring rather than the dihydro-1,4-dioxepin of **1**.

The relative stereochemistry of the above compounds was established *via* pure-absorptive mode 2D

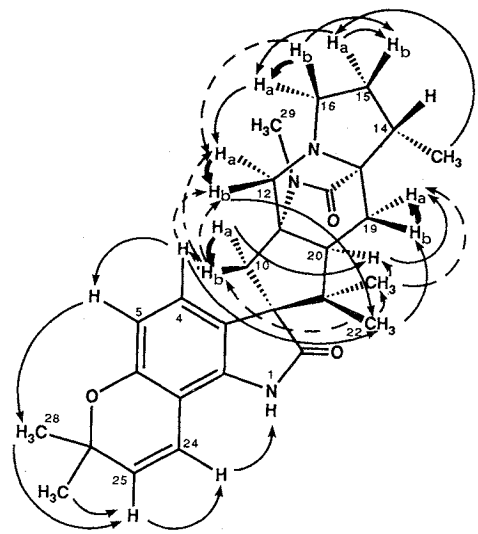
Table 3. EI-MS of compounds **2**~**7** (*m/z* (relative intensity)).

2	463.2488 (4, M^+, $C_{27}H_{33}N_3O_4$), 404 (78), 135 (48), 133 (100)
3	475.2476 (8, M^+, $C_{28}H_{33}N_3O_4$), 416 (30), 147 (40), 145 (100)
4	491.2436 (68, M^+, $C_{28}H_{33}N_3O_5$), 432 (80), 163 (36), 161 (100)
5	477.2602 (2, M^+, $C_{28}H_{35}N_3O_4$), 418 (48), 149 (36), 147 (100)
6	461.2665 (2, M^+, $C_{28}H_{35}N_3O_3$), 402 (42), 149 (36), 147 (100)
7	477.2612 (2, M^+, $C_{28}H_{35}N_3O_4$), 418 (24), 165 (28), 163 (100)

NOE experiments on several of the structural analogs (**4**~**7**). A representative data set is presented for **7** in Fig. 3. The medium intensity NOE's from 12-Hb and 4-H to the 22-H methyl group were critical in establishing the relative stereochemistry of the spiro center. The NOE data also allowed stereo-specific proton assignments of the geminal proton pairs for 10-H, 12-H, 15-H, 16-H, 19-H and 20-H, and one set of geminal methyl groups (22-H and 23-H). Similar sets of NOE results to those presented for **7** in Fig. 3 were obtained for the analogs, indicating the same relative stereochemistry as **7**. Extrapolation to the other analogs and paraherquamide led to the proton asignments as in Table 1 and the relative stereochemistries shown for **1**~**7**. The NOE determined stereochemistry is in agreement with the stereochemistry established by the recently published crystal structure of a synthetic paraherquamide analog[6] and the crystal structure of paraherquamide[1].

Fig. 3. NOE data in CD_2Cl_2 for paraherquamide G (**7**).

The figure depicts ^1H-^1H NOE's of weak (<2.5% of diagonal based on volume integration of diagonal and cross peak), medium (2.5~10%), and strong (>10%) intensities as dashed, solid, and bold arrows, respectively.



Experimental

^1H NMR spectra were recorded at ambient room temperature in CD_2Cl_2 on a Varian XL-400 NMR spectrometer at 400 MHz. Chemical shifts are shown in ppm relative to TMS at 0 ppm using the solvent peak at 5.32 ppm as internal reference. ^{13}C NMR spectra were recorded in CD_2Cl_2 at ambient room temperature on a Varian XL-400 spectrometer at 100 MHz using Waltz-16 proton decoupling. Chemical shifts are given in ppm relative to TMS at 0 ppm using the solvent peak at 53.8 ppm as internal reference. ^1H-^1H chemical shift correlation spectra for **1**~**7** (COSY): Spectra were recorded in CD_2Cl_2 or $(CH_3)_2CO$-d_6 using the standard pulse sequence[7]. Typically, a 2K×2K data set was accumulated in 512 increments with 16 or 32 transients for each value of t_1. The delay time between scans was 1.0 second. ^1H-^{13}C chemical shift correlation spectrum for **1** (HETCOR): Spectra were recorded in CD_2Cl_2 using the standard pulse sequence[8]. A 512×2K data set was accumulated in 128 increments with 400 transients for each value of t_1. The delay time between scans was 1.0 second and the experiment was optimized for $^1J_{CH}=140$ Hz. The related experiment was used to establish long-range connectivities, optimizing for a multiple bond ^{13}C-^1H coupling constant of 7 Hz. The 512×2K data set was accumulated as above with 800 transients for each value of t_1. Pure-absorptive mode 2D NOE effect for **4**~**7** (NOESY): Spectra were recorded in CD_2Cl_2 for dilute (approximately 2.5 mg in 0.5 ml), degassed samples using the standard pulse

sequence with phase-sensitive detection[9]. Typically 2K × 2K data sets were accumulated in 256 increments with 192 transients for each value of t_1. Mixing time was 0.5 second and the delay time between scans (equilibration delay) was 2.5 seconds.

MS were recorded on a Finnigan-MAT MAT212 instrument by EI at 90eV. Exact mass measurements were made on the same instrument at HR by the peak matching method using perfluorokerosene as the internal standard.

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