Bioactive Carotanes from *Trichoderma virens*

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Four new metabolites with carotane skeletons, trichocaranes A (**1**), B (**2**), C (**3**), and D (**4**), were isolated from *Trichoderma virens* and their structures established by the interpretation of NMR and mass spectroscopic data. The trichocaranes significantly inhibited the growth of etiolated wheat coleoptiles: 40% at 10^{-4} M with **1** and **2** and 86% at 10^{-3} M with **3**.

The fungal genus *Trichoderma* is a widespread saprophyte that occurs almost ubiquitously. The species classification is often difficult to determine. Because of the useful secondary metabolites produced by this genus, many strains have received considerable attention as biocontrol agents. Examples include *T. longibrachiatum*[1,2] used to control American leaf-spot disease in coffee, caused by *Mycena citricolor*, and *T. harzianum* and *T. viride*, which are employed to control myriad soil-borne and systemic phytopathogens.

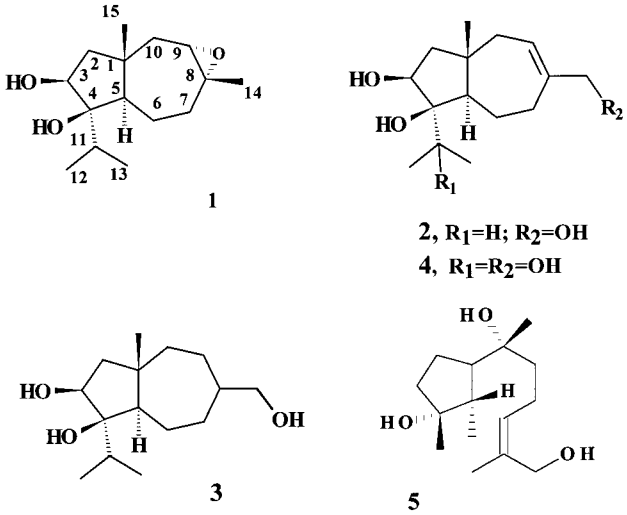
Several secondary metabolites have been elucidated from *Trichoderma* sp.,[1−5] including the *N*-acylated peptides, and the peptibols,[3,4] that have membrane-perturbing and antibiotic activity. The koninginins A−G have also been reported from *T. koningii*,[6−13] as has 6-*n*-pentyl-2*H*-pyran-2-one.[14] The latter also occurs in other *Trichoderma* species and is a potent post-harvest antibiotic. Koninginins A and D are active against *Gaeumannomyces graminis* var. *tritici* and several other soil-borne plant pathogens. Five metabolites have been isolated from *T. harzianum* [three octaketide-derived compounds and two butenolide compounds containing the 3,4-dialkylfuran-2(5*H*)-one nucleus],[15,16] which showed antibiotic activity toward the take-all fungus, *G. graminis* var. *tritici*.

During the course of examining fungi for biogically active natural products, fungi from New Zealand were collected and grown on either semisolid, liquid, or shake fermentation substrate. The fermented fungi were extracted, and bioassay-directed fractionation using the etiolated wheat bioassay yielded pure compounds. We now report the isolation and structure elucidation of four new carotanes, trichocaranes **A**−**D**, in addition to the known cyclonerotriol, from a strain of *Trichoderma virens* Miller.[17]

Results and Discussion

Fermented *T. virens* extract, grown in semisolid fermentation, inhibited wheat coleoptile growth 100%. Fractionation by column chromatography yielded five sesquiterpenoids, four new carotanes (**1**-**4**), and cyclonerotriol (**5**).[17]

Trichocarane A (**1**) showed a molecular ion at *m/z* 254.1836 in the HRMS, in agreement with the molecular formula $C_{15}H_{26}O_3$. Additional peaks in the EIMS at *m/z* 237 $[M - OH]^+$, 219 $[M - OH - H_2O]^+$, and 211 $[M - CH(CH_3)_2]^+$, and strong absorption at 3333 cm^{-1} in the IR spectrum indicated the presence of an isopropyl moiety and two or more hydroxyl groups.



The ^{13}C NMR spectrum confirmed the existence of two hydroxyl groups [C-3 (δ 71.8) and C-4 (δ 83.7)] and an epoxide group [C-8 (δ 60.3) and C-9 (δ 61.2)]. The ^1H NMR spectrum had signals typical of a carotane isopropyl system, two doublets corresponding to H-12 and H-13 (δ 0.94 and δ 0.82) coupled with a septet at δ 1.81. Other three-spin systems in the COSY spectrum of **1** could be established; a signal at δ 4.04 (1H, ddd), a doublet at δ 2.36 (1H, d), and a multiplet at δ 1.57 were assigned to H-3α, C-3-O*H*, and H-2, respectively. The second system was identified by three doublets of doublets at δ 2.74, 2.21, and 1.15, assigned to H-9β, H-10β, and H-10α, respectively. The third group of connected signals at δ 1.32, 1.59, and 2.13, corresponded to a $-CH-CH_2-CH_2-$ system. These data, and the HMQC and HMBC spectra, confirmed the carotane skeleton of **1**, with the hydroxyl groups attached at C-3 and C-4 and the epoxy group between C-8 and C-9. The relative stereochemistry was established based on the observed NOE effects for the most stable conformer of **1**, using PM3 calculations.[18] NOE effects were observed between signals of the methyl groups H-14 and H-15, the epoxide proton H-9, and the protons C-3-O*H* and H-10β. On the other hand, clear NOE effects between signals of the isopropyl group and H-3α and H-5α and between H-3α and H-2α were observed. The relative stereochemistry was in agreement with the previously isolated fungal caro-

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tanes,[19] and the structure of trichocarane A (**1**) was assigned as 8α,9α-epoxy-3β,4β-dihydroxycarotane.

Trichocarane B (**2**) showed a typical mass spectrum for the carotane skeleton, with molecular formula $C_{15}H_{26}O_3$ (*m*/*z* 254.1882) and peaks indicating three hydroxyl groups (*m*/*z* 237 $[M - OH]^+$, 219 $[M - OH - H_2O]^+$, and 201 $[M - OH - 2H_2O]^+$) and an isopropyl moiety *m*/*z* 211 $[M - CH(CH_3)_2]^+$. The presence of hydroxyl groups was confirmed by IR. The ^1H and ^{13}C NMR spectra of **2** showed signals for the carotane five-membered ring with the same substitution as compound **1**, where the hydroxymethine group at C-3 (δ 72.5, H-3; δ 4.05, dd), the quaternary hydroxyl group at C-4 (δ 84.4), and the isopropyl group with the typical septet (H-11, δ 1.82, sp) and two doublets of 3H (H-12, δ 0.97, and H-13, δ 0.88,) were assigned. The substitution of the seven-membered ring was determined as a $\Delta^{8,9}$ double bond (C-8, δ 142.2; C-9, δ 124.2, H-9, δ 5.63, br d) and a hydroxymethylene group at C-14 (δ 69.5; H-14, δ 4.00, s). The overall skeletal features were confirmed by 2D NMR analysis involving COSY, HMQC, and HMBC experiments (see Supporting Information). The spectroscopic data of **2** are in good agreement with those previously reported for 14-hydroxy CAF-603, obtained by hydrolysis of 14-hydroxy CAF-603 oleate isolated from *T. virens*.[20] Thus, the structure of trichocarane B (**2**) was determined as 3β,4β,14-trihydroxycarota-8-ene.

Trichocarane C (**3**) showed spectral patterns similar to those of **2**. The HRMS gave a molecular ion at *m*/*z* 256.2059, corresponding to molecular formula $C_{15}H_{28}O_3$. The EIMS exhibited additional peaks at *m*/*z* 239 $[M - OH]^+$, 221 $[M - OH - H_2O]^+$, 203 $[M - OH - 2H_2O]^+$, and 211 $[M - CH(CH_3)_2]^+$, following a fragmentation pattern similar to compound **2**. The higher degree of saturation of compound **3**, the absence of double-bond signals in its ^1H and ^{13}C NMR spectra, and the H-14 signal (δ 3.43, dd and δ 3.37, d) coupled with H-8 (δ 1.86, m) were the only differences for compound **3** compared to compound **2**. Thus, the structure of trichocarane C (**3**) was established as 3β,4β,14-trihydroxycarotane.

The HRMS of trichocarane D (**4**) showed a molecular ion at *m*/*z* 270.1811 ($C_{15}H_{26}O_4$) and a fragmentation pattern analogous to the other isolated carotanes (*m*/*z* 253 $[M - OH]^+$, 235 $[M - OH - H_2O]^+$, 217 $[M - OH - 2H_2O]^+$, and 211 $[M - COH(CH_3)_2]^+$. The presence of hydroxyl groups was confirmed by IR. The ^1H and ^{13}C NMR spectra were very similar to those of compound **2**. The only difference between the two compounds was the presence of an additional hydroxyl group at C-11. The ^1H NMR spectrum of compound **4** does not show the typical septet signal of H-11, and the H-12 and H-13 signals suffered deshielding effects and collapsed to singlets (δ 1.20, s and δ 1.25, s). The ^{13}C NMR spectrum contained four signals of hydroxy-substituted carbons (δ 73.0, C-3; δ 85.4, C-4; δ 75.6, C-11; and δ 69.4, C-14). The structure of **4** was confirmed from analysis of the COSY, HMQC, and HMBC spectra (see Supporting Information). The relative stereochemistry was established based on the observed NOE for the most stable conformer of **4**. Thus, the structure of trichocarane D (**4**) was determined as 3β,4β,11,14-tetrahydroxycarota-8-ene.

Because secondary metabolite production, or lack thereof, is directly dependent upon the fermentation substrate, three different fermentation protocols were followed: semisolid, liquid, and liquid-shake.[21]

The etiolated wheat bioassay is rapid (less than 24 h) and sensitive to a wide range of bioactive substances, including plant growth regulators, herbicides,[22] antimicrobials, mycotoxins, and assorted pharmaceuticals.[23] Crude extracts from *Trichoderma virens* showed high degrees of bioactivity in the etiolated wheat bioassay: semisolid and liquid fermentation produced 100% inhibition in the bioassay, while shake fermentation extracts were inactive.

Etiolated wheat coleoptile growth was significantly ($p < 0.01$) inhibited (40%) with 10^{-4} M solutions of **1** and **2**. However, **2** inhibited 19% at 10^{-5} M, and **3** inhibited 86% at 10^{-3} M, all relative to controls. But **4** was inactive in the bioassay. Insufficient material was available to test either **1** or **2** at 10^{-3} M. The functionalities between C-8 and C-9 and at C-11 appear to direct the biological activity, but absolute proof of this observation will have to wait until a series of derivatives can be synthesized and tested.[24]

Experimental Section

General Experimental Procedures. ^1H NMR and ^{13}C NMR spectra were made at 399.952 and 100.577 MHz, respectively, on a Varian UNITY-400 spectrometer and with $CDCl_3$ as solvent. The resonances of residual $CHCl_3$ at δ_H 7.25 and $CDCl_3$ at δ_C 77.00 ppm were used as internal reference for ^1H and ^{13}C spectra, respectively. Mass spectra were obtained using a VG 1250 or a Kratos MS-80-RFA instrument at 70 eV. The IR spectra were recorded on a Bio-Rad FTS-7. Optical rotations were determined using a Perkin-Elmer polarimeter model 241 set on the sodium D line. Column chromatography was performed on Si gel (35−75 mesh), and TLC analysis was carried out using aluminum precoated Si gel plates. For HPLC, LiChrosorb silica 60 was used in the normal-phase mode using differential refractometer (RI) and UV detectors, with a Hitachi L-6020A HPLC instrument. All solvents were spectral grade or distilled from glass prior to use.

Fungal Material. A strain of *T. virens* Miller. Giddens and Foster (von Arx) [formerly *Gliocladium virens* Miller], was collected (from strawberry roots) in Auckland, New Zealand. *T. virens* is characterized by large obovid conidia, predominantly effuse conidiation, with broad conidiophores, sparingly branched, and fertile to the apex.[25] A voucher specimen is deposited at Mercer University with the code NZ-160. The fungus was cultured on potato-dextrose agar slants at 26 °C for 10 days and then maintained at 5 °C.

Semisolid Fermentation. The fungus was transferred to Fernbach flasks (2.8 L), each containing 100 g of shredded wheat, 200 mL of Difco mycological broth (pH 4.8), 2% yeast extract, and 20% sucrose[26] for metabolite production. Inoculated flasks were incubated in the laboratory at 24 °C for 19 days.

Still Fermentation: Fungal plugs were transferred to Fernbach flasks (2.8 L), each containing 200 mL of potato-dextrose broth. Inoculated flasks were incubated in the laboratory at 24 °C for 19 days.

Shake Fermentation. This preparation was identical to still fermentation, but shaken on a rotary shaker at 80 rpm.

Extraction and Isolation. After incubation, 300 mL of acetone was added to each semisolid fermentation flask, the mycelia and substrate were macerated with a Super Dispax homogenizer, and the suspension was strained through cheesecloth to remove the pulp. The liquid was then filtered through Whatman no. 1 filter paper on a Büchner funnel. The clarified filtrate was reduced under vacuum at 50 °C to yield an aqueous phase. This was extracted twice with ethyl acetate, each volume of solvent was equal to twice that of the aqueous portion. Combined ethyl acetate extracts were dried over anhydrous sodium sulfate and reduced to a small volume under vacuum. Both the liquid and the liquid shake were partitioned against ethyl acetate (×3), dried over anhydrous sodium sulfate, then reduced in volume in vacuo.

The extract from semisolid fermentation (2.1 g) was separated by column chromatography on Si gel using *n*-hexane−EtOAc 40% to afford 12 fractions. Fraction D (150 mg) was chromatographed using Si gel, eluting with $CHCl_3$−EtOAc

20%. The fractions of low polarity were combined and chromatographed using preparative TLC ($CHCl_3$−EtOAc 20%). Product **1** (5.5 mg) was isolated from HPLC fractions eluted with hexane−acetone 35% with a Hibar Si 60 (Merck) 10-μm column, at 3 mL min^{-1} flow rate and an RI detector. Fractions F and G were combined (120 mg) and chromatographed using Si gel and eluted with hexane−acetone 20%, collecting fractions of 25 mL. Fractions 29−35 (30 mg) were chromatographed using preparative TLC ($CHCl_3$−acetone 15%, developed twice). A mixture of two products (**2** and **3**) was obtained, which was separated using HPLC with a Hibar Si 60 (Merck) 5-μm column, at 1 mL min^{-1} flow rate and an RI detector, yielding 6 mg of **2** and 4 mg of **3**. Fraction L (50 mg) was separated using repeated preparative TLC with hexane−acetone 30% as eluent, yielding compounds **4** (19 mg) and **5** (8 mg).

Trichocarane A (1): colorless oil; $[\alpha]^{25}_D$ −12.7° (*c* 0.1, $CHCl_3$); IR (neat, KBr) ν_{max} 3333 (OH), 2949 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.04 (1H, ddd, J = 7.4, 5.6, 1.5 Hz, H-3α), 2.74 (1H, dd, J = 7.2, 7.2 Hz, H-9β), 2.46 (1H, s,C_4−OH), 2.36 (1H, d, J = 5.6 Hz, s, C_3−OH), 2.21 (1H, dd, J = 13.7, 7.2 Hz, H-10β), 2.13 (1H, ddd, J = 14.3, 5.9, 2.2 Hz, H-7β), 1.81 (1H, sp, J = 6.8 Hz, H-11), 1.59 (2H, m, H-6), 1.57 (2H, m, H-2), 1.37 (1H, m, H-7α), 1.34 (3H, s, H-14), 1.32 (1H, m, H-5α), 1.19 (3H, s, H-15), 1.15 (1H, dd, J = 13.7, 7.2 Hz, H-10α) 0.94[a] (3H, d, J = 6.8 Hz, H-12), 0.82[a] (3H, d, J = 6.8 Hz, H-13), [a](these signals may be interchanged); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 83.7 (s, C-4), 71.8 (d, C-3), 61.2 (d, C-9), 60.3 (s, C-8), 59.2 (d, C-5), 49.5 (t, C-2), 44.1 (s, C-1), 42.7 (t, C-10), 36.4 (t, C-7), 34.7 (d, C-11), 23.5 (q, C-14), 20.6 (q, C-15), 19.9 (t, C-6), 17.7[b] (q, C-13), 16.9[b] (q, C-12), [b](these signals may be interchanged); EIMS, m/z 254 $[M]^+$ (1), 237 $[M - OH]^+$ (3), 219 $[M - OH - H_2O]^+$ (9), 211 $[M - CH(CH_3)_2]^+$ (17), 193 (88), 175 (36), 165 (29), 147 (43), 133 (49), 125 (51), 121 (100), 107 (52), 95 (66), 81 (66), 71 (91); HREIMS m/z 254.1835 (calcd for $C_{15}H_{20}O_4$, 254.1882).

Trichocarane B (2): colorless oil; $[\alpha]^{25}_D$ −28.0° (*c* 0.2, $CHCl_3$); IR (neat, KBr) ν_{max} 3355 (OH), 3287 (OH), 2921 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.63 (1H, br d, J = 8.9 Hz, H-9β), 4.05 (1H, dd, J = 7.4, 1.5 Hz, H-3α), 4.00 (2H, s, H-14), 2.28 (1H, br ddd, J = 15.1, 5.3, 2.2 Hz, H-7β), 2.13 (1H, dd, J = 14.7, 8.9 Hz, H-10β), 2.00 (1H, br ddd, J = 15.1, 12.0, 2.2 Hz, H-7α), 1.85 (1H, br d, J = 14.7 Hz, H-10α), 1.82 (1H, sp, J = 6.8 Hz, H-11), 1.62 (3H, m, H-2, H-6α), 1.51 (1H, dd, J = 11.6, 1.4 Hz, H-5α), 1.43 (1H, ddd, J = 12.1,12.0, 2.2 Hz, H-6β), 1.02 (3H, s, H-15), 0.97[a] (3H, d, J = 6.8 Hz, H-12), 0.88[a] (3H, d, J = 6.8 Hz, H-13), [a](these signals may be interchanged); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 142.2 (s, C-8), 124.2 (d, C-9), 84.4 (s, C-4), 72.5 (d, C-3), 69.5 (t, C-14), 58.4 (d, C-5), 50.4 (t, C-2), 42.6 (t, C-10), 42.1 (s, C-1), 35.3 (d, C-11), 30.4 (t, C-7), 21.2 (t, C-6), 21.2 (q, C-15), 17.8[b] (q, C-13), 17.0[b] (q, C-12), [b](these signals may be interchanged); EIMS, m/z 254 $[M]^+$ (3), 237 $[M - OH]^+$ (13), 219 $[M - OH - H_2O]^+$ (38), 211 $[M - CH(CH_3)_2]^+$ (42), 201 (28), 193 (88), 175 (55), 165 (21), 147 (58), 133 (35), 123 (13), 121 (100), 105 (39), 93 (48), 81 (34), 71 (56); HREIMS m/z 254.1893 (calcd for $C_{15}H_{20}O_4$, 254.1882).

Trichocarane C (3): colorless oil; $[\alpha]^{25}_D$ −11.1° (*c* 0.15, $CHCl_3$); IR (neat, KBr) ν_{max} 3358 (OH), 2924, 2855 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.03 (1H, m, H-3α), 3.43 (1H, dd, J = 10.3, 5.9 Hz, H-14a), 3.37 (1H, dd, J = 10.3, 6.6 Hz, H-14b), 2.38 (1H, m, O*H* on C-3), 1.86 (1H, m, H-8), 1.79 (1H, sept, J = 6.8 Hz, H-11), 1.58 (2H, m, H-2), 1.16 (3H, s, H-15), 0.96[a] (3H, d, J = 6.8 Hz, H-12), 0.86[a] (3H, d, J = 6.8 Hz, H-13), [a] (these signals may be interchanged); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 83.9 (s, C-4), 72.6 (d, C-3), 68.9 (t, C-14), 55.5 (d, C-5), 51.6 (t, C-2), 41.5 (d, C-8), 39.9 (t, C-10), 43.9 (s, C-1), 35.1 (d, C-11), 30.1[b] (t, C-7), 27.2[b] (t, C-9), 23.8[c] (q, C-15), 22.9[c] (t, C-6), 17.7[d] (q, C-13), 17.0[d] (q, C-12), [b,c,d](these signals may be interchanged); EIMS, m/z 256 $[M]^+$ (7), 239 $[M - OH]^+$ (5), 221 $[M - OH - H_2O]^+$ (19), 213 $[M - CH(CH_3)_2]^+$ (57), 195 (76), 177 (100), 165 (10), 159 (31), 149 (42), 135 (24), 123 (38), 121 (31), 109 (32), 107 (36), 95 (34), 93 (46), 81 (63), 71 (60); HREIMS m/z 256.2059 (calcd for $C_{15}H_{20}O_4$, 256.2038).

Trichocarane D (4): colorless oil; $[\alpha]^{25}_D$ −8.8° (*c* 1.8, $CHCl_3$); IR (neat, KBr) ν_{max} 3411 (hydroxyl group), 1681 (double bond), 1110 (ether) cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.60 (1H, br d, J = 8.8 Hz, H-9β), 4.19 (1H, br d, J = 7.2 Hz, H-3α), 3.98 (3H, s, H-14), 2.23 (1H, ddd, J = 15.4, 5.0, 1.8 Hz, H-7β), 2.13 (1H, dd, J = 15.0, 8.8 Hz, H-10β), 1.98 (1H, ddd, J = 15.4, 12.1, 2.3 Hz, H-7α), 1.92 (1H, m, H-6α), 1.87 (1H, br d, J = 15.0 Hz, H-10α), 1.67 (1H, dd, J = 14.0, 0.8 Hz, H-2β), 1.57 (1H, dd, J = 14.0, 7.2 Hz, H-2α), 1.54 (1H, d, J = 11.7 Hz, H-5α), 1.46 (1H, br dd, J = 13.8, 12.1 Hz, H-6β), 1.25[a] (3H, s, H-13), 1.20[a] (3H, s, H-12), 1.00 (3H, s, H-15), [a](these signals may be interchanged); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 142.0 (s, C-8), 123.6 (d, C-9), 85.4 (s, C-4), 75.6 (d, C-11), 73.0 (d, C-3), 69.4 (t, C-14), 57.1 (d, C-5), 49.6 (t, C-2), 42.6 (s, C-1), 42.4 (t, C-10), 30.5 (t, C−7), 22.3 (t, C-6), 25.6[b] (q, C-13), 24.7[b] (q, C-12), 21.2 (q, C-15), [b](these signals may be interchanged); EIMS, m/z: 270 $[M]^+$ (2), 253 $[M - OH]^+$ (8), 235 $[M - OH - H_2O]^+$ (35), 217 $[M - OH - 2H_2O]^+$ (44), 211 $[M - CH(CH_3)_2]^+$ (34), 193 (100), 175 (66), 165 (24), 161 (25), 147 (56), 133 (45), 121 (82), 119 (34), 107 (34), 93 (39), 81 (37), 69 (24); HREIMS m/z 270.1811(calcd for $C_{15}H_{20}O_4$, 270.1831).

Bioassay. Wheat seeds (*Triticum aestivum* L. cv. Wakeland) were sown on fine, moist vermiculite in trays and grown in the dark at 22 ± 1 °C for 4 days.[27] The etiolated seedlings were removed from the trays, and the roots and caryopsis were removed from the shoots. The latter were placed in a Van der Weij guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassay. All manipulations were performed under a green safelight.[28] Crude extracts, fractions, or pure compounds to be assayed for biological activity were added to test tubes (approximately 20 μL/tube) and evaporated under nitrogen to dryness. The assay was duplicated. Phosphate-citrate buffer (2 mL) containing 2% sucrose[28] at pH 5.6 was added to each test tube. After the placement of 10 coleoptiles in each test tube, the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles were measured by projecting their images (× 3) from a photographic enlarger.[29] Data were statistically analyzed.[30]

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Supporting Information Available: COSY and HMBC connectivities (**1**, **2**, **4**) and observed NOE enhancement (**1**, **4**).

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