Constituents of *Ardisia japonica* and Their in Vitro Anti-HIV Activity

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Received August 10, 1995[⊗]

As part of our screening of anti-AIDS agents from medicinal plants, the MeOH extract of the aerial parts of *Ardisia japonica* was tested, and it showed moderate in vitro anti-HIV activity. Reexamination to identify the compounds responsible for the anti-HIV activity revealed several known compounds and a new triterpenoid saponin (**4**) whose structure elucidation was accomplished by ^{1}H−^{1}H correlation spectroscopy (COSY, HOHAHA, ROESY) and ^{1}H-^{13}C heteronuclear correlation (HETCOR) NMR experiments. All of the isolated compounds were tested and, although none of the triterpenoid saponins was active, bergenin and norbergenin showed weak anti-HIV activity.

Introduction

Ardisia japonica (Thunb.) Bl. (Myrsinaceae), in the form of a decoction of the roots, is widely used in traditional Chinese medicine to stop cough and uterine bleeding.[1] Medicinal use of 16 other species of *Ardisia* has also been reported.[1]

Our previous studies on *A. japonica* resulted in the isolation and identification of three new triterpenoid glycosides (**1**−**3**) characterized by oleane-type aglycons.[2] During continuing studies on anti-HIV activity of plant metabolites,[3−6] we found that the crude MeOH extract from the aerial parts of *A. japonica* showed moderate anti-HIV activity in a preliminary test. More detailed investigation afforded the previously isolated saponins **1**−**3**, a novel triterpene glycoside **4**, and the known compounds norbergenin (**5**), bergenin (**6**), and tri-*O*-methylnorbergenin (**7**).

Bergenin and related compounds are dihydroisocoumarin derivatives characterized by a β-D-glucosyl residue C-linked to a hydroxylated phenylcarboxylic acid ortho to the carboxyl group. In addition, the carboxyl group is esterified with the C-2 hydroxyl group of the glucosyl moiety to form a δ-lactone ring. Bergenin, the most prominent and widely distributed representative of this class of compounds, was isolated first from rhizomes of three *Bergenia* species.[7] Recently, antiulcer effects[8] and hypolipidemic activity[9] in rats have been reported for bergenin.

Various biological activities have been described for triterpenoid saponins,[10] however, little is known regarding antiviral activity, which was reported for the glycoside glycyrrhizin,[11] glycyrretinic acid,[12] and some other triterpenoids that were found to inhibit the multiplication of some DNA viruses.[13] Recently, anti-HIV activity was reported for some triterpenes.[14] The anti-HIV activity of all the isolated compounds from *A. japonica* was tested. Although triterpene saponins did not inhibit HIV replication, bergenin and norbergenin showed significant anti-HIV activity.

In this paper we report the isolation and structure elucidation of the novel triterpene glycoside **4** by 2D ^{1}H−^{1}H and ^{1}H−^{13}C shift correlation spectroscopy and the in vitro HIV inhibition in infected C8166 cells by bergenin and norbergenin.

Results and Discussion

The aerial parts of *A. japonica* were extracted successively with petroleum ether, $CHCl_3$, and MeOH. The MeOH extract was partitioned with *n*-BuOH and H_2O to afford the *n*-BuOH-soluble portion, which was subjected to Sephadex LH-20 column chromatography, followed by DCCC ($CHCl_3$-MeOH-H_2O-*n*-PrOH-EtOH, 9:6:8:1:8, descending mode) and then reversed-phase HPLC to give four pure compounds (**1**−**4**).

Compound **4** had a molecular formula $C_{58}H_{94}0_{26}$, as determined by ^{13}C and ^{13}C DEPT NMR data and FABMS analysis in negative ion mode. The FABMS of **4** showed the $[M - H]^-$ ion at *m*/*z* 1205 and fragments at *m*/*z* 1043 $[(M - H) - 162]^-$, *m*/*z* 1059 $[(M - H) - 146]^-$ (cleavage of a deoxyhexose unit with or without the glycosidic oxygen), *m*/*z* 1073 $[(M - H) - 132)]^-$ due to the loss of a pentose unit, *m*/*z* 911 $[(M - H) - (162 + 132)]^-$ due to the loss of pentose and deoxyhexose units, and *m*/*z* 603 $[(M - H) - (146 + 132 + 162 + 162)]^-$. The ^{13}C and DEPT ^{13}C-NMR spectra showed 58 signals, of which 28 were assigned to the saccharide portion and 30 to a triterpene moiety.

^{1}H- and ^{13}C-NMR signals assigned to the pentacyclic nucleus of the aglycon were superimposable with those reported for cyclamiretina A.[13] Detailed analysis of NMR sugar data by comparison with those of **1**−**3**[2] indicated the attachment of the glycosidic chain at C-3 of the aglycon moiety and the presence of an additional

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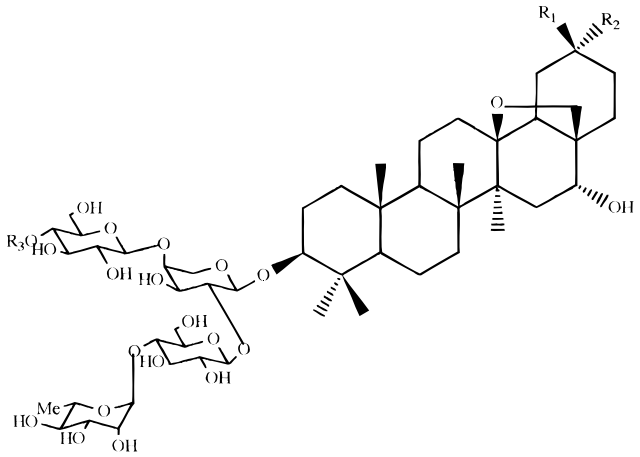
[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1996.

S0163-3864(96)00074-2 CCC: $12.00



1	R_1=CHO	R_2=CH_3	R_3=H
2	R_1=CHO(CH_3)$_2$	R_2=CH_3	R_3=H
3	R_1=CH_3	R_2=COOH	R_3=H
4	R_1=CHO	R_2=CH_3	R_3=

pentose unit. The 3β-OH substitution was evident from the chemical shift and from the *J* value of the proton ascribable to C-3 at δ 3.17 (dd, J = 11 and 4.5 Hz). Anhydrous acidic methanolysis of **4** gave methyl arabinoside, methyl rhamnoside, methyl xyloside, and methyl glucoside in a 1:1:1:2 ratio.

The structure of the oligosaccharide unit was determined by 2D-NMR spectroscopy. Even at highfield (500 MHz) the 1D sugar spectral region of **4** was complex, as most of the signals were found between δ 5.29 and 3.00 and were overlapped with the aglycon signals. 2D HOHAHA spectroscopy experiments[16] allowed resolution of the overlapped spectra of oligosaccharides into a subset of individual monosaccharide spectra. In the 2D HOHAHA spectrum of **4** the anomeric proton signal ascribable to an α-L-arabinose (H-1′, δ 4.53, J = 5.2 Hz) showed connectivities to three methines (δ 4.06, 3.92, and 3.86). The coherence transfer to methylene H-5′ was not obtained because of the small coupling constants between H-4′and H-5′.[2] As in the HOHAHA method, the cross peaks represent both direct and relayed connectivities; we also recorded a 2D COSY-90 spectrum that established the proton sequence within this sugar fragment as H-1 (δ 4.53), H-2 (δ 3.86), H-3 (δ 3.92), and H-4 (δ 4.06) (Table 1). Similar results from the HOHAHA and COSY experiments for all the other sugar residues (Table 1) allowed complete sequential assignments for all proton resonances starting from the anomeric proton signals.

HETCOR experiments, which correlated all proton resonances with those of each corresponding carbon (Table 1), permitted assignments of the interglycosidic linkages by comparing the carbon chemical shift observed with those of the corresponding methyl pyranosides and taking into account the known effects of glycosidation.[17] The absence of any ^{13}C glycosidation shift for xylopyranosyl and rhamnopyranosyl residues suggested that these sugars were terminal units, while glycosidation shifts of C-2 (+7 ppm) and C-4 (+7) of arabinose and of C-4 (ca. +6 ppm) of the two glucose units established the presence of an arabinopyranosyl residue glycosylated at C-2 and C-4 and two C-4 glycosylated glucopyranosyl units. The location of each sugar unit was deduced from a 2D NOESY experiment which showed a cross peak between the signals at δ 4.53 (H-1′ of the arabinose) and δ 3.17 (H-3 of the aglycon moiety), and other key correlation peaks (Table 2) between anomeric protons and protons linked to glycosylated carbons.

Although ^{1}H- and ^{13}C-NMR data (Table 2) indicated the β-configuration at the anomeric positions for the glucopyranosyl units (J_{H1-H2} = 7.5 Hz) and the xylopyranosyl unit (J_{H1-H2} = 7.0 Hz), and the α-configuration for the rhamnopyranosyl unit (Table 2) (J_{H1-H2} = 1.5 Hz), for the L-arabinopyranosyl unit it was possible to deduce only the pyranose form from the ^{13}C-NMR data.[18] No further information on the anomeric configurations could be drawn from the ^{1}H NMR and ^{13}C NMR. The value of J_{H1-H2} coupling constant (5.2 Hz), midway between that observed for methyl-β-L-arabinopyranoside (4 Hz) and methyl-α-L-arabinopyranoside (8

Table 1. ^{1}H- and ^{13}C-NMR Data of the Sugar Portion of **4** in CD_3OD[a]

sugar		δC	δH, *J* in Hz
ara	1	105.3	4.53, d, 5.2
	2	79.8	3.86, dd, 5.2, 8.0
	3	72.5	3.92, dd, 8.0, 3.0
	4	76.4	4.06, m
	5	64.0	4.11, dd, 2.0, 12.0
			3.62, dd, 2.5, 12.0
glu I	1	104.5	4.60, dd, 7.5
	2	77.0	3.27, dd, 7.5, 9.5
	3	77.9	3.48, t, 9.5, 9.5
	4	78.5	3.44, t, 9.5, 9.5
	5	77.3	3.37, m
	6	62.5	3.90, dd, 3.5, 12.0
			3.70, dd, 5.0, 12.0
rha	1	101.7	5.29, d, 1.5
	2	71.8	3.91, dd, 1.5, 2.5
	3	72.2	3.77, dd, 2.5, 9.0
	4	74.2	3.42, dd, 9.0, 9.0
	5	70.3	4.12, dd, 9.0, 6.5
	6	17.0	1.28, d, 6.5
glu II	1	103.5	4.68, dd, 7.5
	2	76.6	3.41, dd, 7.5, 9.5
	3	77.9	3.45, t, 9.5, 9.5
	4	77.9	3.49, t, 9.5, 9.5
	5	77.3	3.39, m
	6	62.5	3.85, dd, 3.5, 12.0
			3.66, dd, 5.0, 12.0
xyl	1	108.0	4.38, d, 7.0
	2	75.0	3.24, dd, 7.0, 9.0
	3	77.8	3.41, m
	4	70.5	3.52, m
	5	67.3	3.20, t
			3.87 dd, 10.5, 4.0

[a] Assignments based on 2D COSY, 2D HOHAHA, and HETCOR experiments. ^{1}H-^{1}H coupling constants in the sugar spin system were measured from COSY and HOHAHA spectra and are reported in Hz.

Table 2. Selected Data from NOESY Experiments of **4** in CD_3OD[a]

connectivities between	
proton	^{1}H anomeric
3.17 (H-3 aglycone)	4.53 (H-1 arabinose)
3.86 (H-2 arabinose)	4.60 (H-1 glucose I)
3.44 (H-4 glucose I)	5.29 (H-1 rhamnose)
4.06 (H-4 arabinose)	4.68 (H-1 glucose II)
3.49 (H-4 glucose II)	4.38 (H-1 xylose)

[a] The experiments were optimized for dipolar couplings with a mixing time of 200 msec.

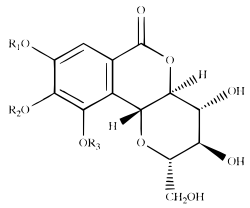
Table 3. Anti-HIV-1 Activity of **5**-**7**

compound	EC_{50}[a]	TC_{50}[b]	S.I.[c]
5	20	>500	>25
6	40	>1000	>25
7	200	250	1.25

[a] EC_{50} = concentration (μg/mL) that reduces by 50% the production of gp120 in infected C8166 cells. [b] TC_{50} = concentration (μg/mL) that causes 50% cytotoxicity to uninfected C8166 cells. [c] S.I. = selectivity index.

Hz),[16−17] has been reported not to be diagnostic on its own, owing to the high conformational mobility of arabinopyranosides ($^4C_1 \leftrightarrow {}^1C_4$).[17] Thus, as we previously reported for **1**−**3**,[2] only on the basis of the observed NOE effects, was it possible to establish an α-L-arabinopyranoside in rapid conformational exchange. Therefore, the structure cyclamiretin A 3β-O-[-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranoside] was assigned to **4**.

Compounds **5**−7, obtained by subjecting the final Sephadex LH-20 fractions to reversed-phase HPLC, were identified, respectively, as norbergenin, bergenin, and tri-*O*-methylnorbergenin, by comparing their NMR data with those reported in the literature.[20- 21]



	R_1	R_2	R_3
5	H	H	H
6	H	CH_3	H
7	CH_3	CH_3	CH_3

As a part of our program for screening natural compounds for anti-HIV activity, the pure compounds isolated from *A. japonica* were tested in C8166 cells infected with HIV-1_{MN}. Although none of the triterpenoid saponins was found to be effective against HIV in this test system, interesting results were observed for bergenin (**6**) and norbergenin (**5**). The results of antiviral activities are presented in Table 3. The most active compound was norbergenin, which exhibited an in vitro EC_{50} value of 20 μg/mL and a TC_{50} value of over 500 μg/mL with a selectivity index above 25.

Although similar in selectivity index, bergenin elicited a lower potency (EC_{50} = 40 μg/mL), and tri-*O*-methylnorbergenin (**7**) was only slightly active. Comparison of the anti-HIV activities of **5**−**7** suggests that the substituents at C-3, C-4, and C-5 of the galloyl moiety play an important role in the demonstration of anti-HIV activity. It was evident that replacement of the 4-OH group of the galloyl moiety with $-OCH_3$ in **6** reduced the EC_{50} by 50%, whereas *O*-methylation of the three phenolic functions in **7** greatly reduced anti-HIV activity.

A detailed study of the mechanism of action revealed that, like dextran sulfate, norbergenin was more effective when added prior to or at the time of virus infection. The compound neutralized virus infectivity by more than 99%, when incubated with virus, for 60 min at 37 °C, suggesting that it inhibited at an early stage of the virus infection. Further experiments confirmed that it inhibited the binding of gp120 to sCD4 in a dose-dependent manner (Figure 1).

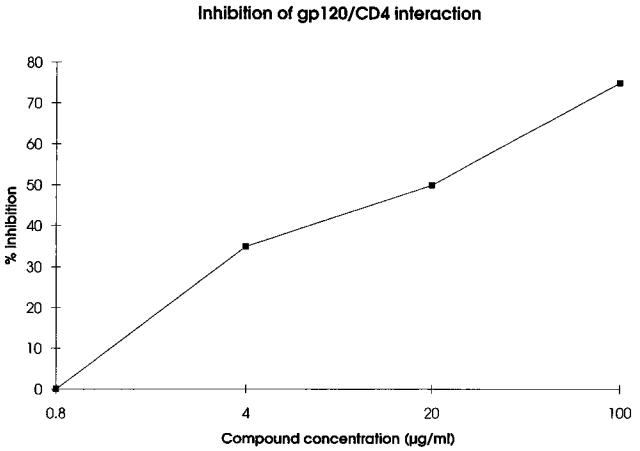


Figure 1. Inhibition of gp120/CD4 interaciton by norbergenin (**5**).

Table 4. Inhibition of Antibody 358 (Directed Against the V3 Loop of gp120) Interaction with Gp120[a]

compound	concn (mg/mL)	inhibition (%)
5	400	84
	80	69
	16	57
	3.2	33
6	500	92
	100	84
	20	62
	4	39
	0.8	0
dextran sulfate	20	78
	4	60
	0.8	33

[a] 96-well plates were coated with recombinant gp120 (1ng/well) in PBS at room temperature for 12 h. After washing with PBS, the wells were blocked with 10% calf serum and incubated with different concentrations of compounds for 15 min before adding appropriate dilutions of antibody. The mixture was incubated at 37 °C for 3−4 h, washed with PBS containing Tween 20 (0.1%), and incubated for another 1.5 h with anti-mouse Ig conjugated with horseradish peroxidase. The antibody binding was detected by using substrate OPD. The percent inhibition was calculated from linear logarithmic plots using three dilutions of antibody, with no compound added.

Some degree of specificity in the interaction of bergenin and norbergenin with gp120 was apparent from their ability to prevent binding of monoclonal antibody ADP358, known to recognize the V3 loop and interfere with gp120-CD4 interaction (Table 4). The compounds had no effect on the binding of monoclonal antibodies ADP360 and ADP323 to the N- and C-terminal, respectively, of gp120. These compounds inhibit infection by binding irreversibly to the V3 loop of gp120 and thereby neutralize viral infectivity.

It was interesting to observe that, although the inhibition of antibody ADP358 binding to gp120 by compound **6** (diluted in PBS) was better than that of **5**, the inhibition of infection in cell cultures was definitely less. The effect of non-specific proteins on the inhibitory effects of related compounds has been observed previously.[22] It is possible that compound **5**, though similar in structure, is slightly more specific than **6** and retains its activity better in the presence of calf serum proteins. This surmise has subsequently been confirmed by the observation that inhibition of antibody binding is re-

duced when dilutions are made in RPMI containing calf serum (data not shown).

Experimental Section

General Experimental Procedures. Bruker WH-250 Spectrospin or Bruker AMX-500 spectrometer, equipped with a Bruker X-32 computer using the UXNMR software package, were used for NMR measurements. Two-dimensional homonuclear proton chemical shift correlation (COSY) experiments were measured by employing the conventional pulse sequence. The 2D HOHAHA experiment was performed in the phase-sensitive mode (TPPI) using an MLEV-17 sequence for mixing. The NOESY experiment was performed in the phase-sensitive mode (TPPI). The HETCOR experiment was performed on a data matrix 512 × 1024, using a CH coupling of 135 Hz and relaxation delay of 1.5 s. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. FABMS were recorded in a glycerol matrix in the negative ion mode on a VG ZAB instrument (Xe atoms of energy of 2−6 KV). Droplet counter current chromatography (DCCC) was performed on an apparatus manufactured by Buchi, equipped with 300 tubes. GLC analyses were performed on a Supelco SP200 capillary column (30 m, i.d. 0.32 mm, film thickness 0.25 mm, carrier gas He, 5 mL min^{-1}, 156 °C).

Plant Material. The plant *Ardisia japonica* (Myrsinaceae) was collected at Suzhou, Jiang-Su Province, China. A voucher sample of the plant was deposited at the herbarium of the Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli "Federico II".

Extraction and Isolation. The air-dried leaves (250 g) were defatted with petroleum ether and $CHCl_3$ and then extracted with MeOH to give 15 g of residue. Part of the MeOH extract (7 g) was partioned between *n*-BuOH and H_2O to afford an *n*-BuOH-soluble portion (4 g) that was chromatographed on a Sephadex LH-20 column (100 × 5 cm), with MeOH as the eluent.

Fractions (8 mL each) were collected and checked by TLC (Si gel plates, *n*-BuOH-HOAc-H_2O 60:15:25). Fractions 16−24 (600 mg each) containing the crude glycosidic mixture were further purified by DCCC with $CHCl_3$−MeOH−H_2O−*n*-PrOH−EtOH (9:6:8:1:8) in which the stationary phase consisted of the higher phase (descending mode, flow 10 mL/h). Compounds **1**−**3** were obtained as described previously.[2] Pure **4** (11 mg, t_R = 13 min) was obtained by HPLC on C_{18} μ-Bondapak column (30 cm × 7.8 mm) with MeOH−H_2O (3:2), flow 3 mL/min; Sephadex LH-20 fractions 44−60 containing bergenin (**6**), norbergenin (**5**), and tri-*O*-methylnorbergenin (**7**) were chromatographed over μ-Bondapak C_{18} using MeOH-H_2O, 3:7 as eluent to yield the pure **5** (15 mg, t_R = 10 min), **6** (20 mg, t_R = 13 min), and **7** (10.5 mg, t_R = 18 min).

Methanolysis of Compound 4, Carbohydrate Constituents. A solution of the compound (2 mg) in anhydrous 2 N HCl-MeOH (0.5 mL) was heated at 80 °C in a stoppered reaction vial for 12 h. After cooling, the solution was neutralized with Ag_2CO_3 and centrifuged, then the supernatant was evaporated to dryness under N_2. The residue was reacted with TRISIL-Z (Pierce) and analyzed by GLC. Retention times were identical to those of the authentic methyl sugars.

Compound 4: $[\alpha]^{25}_D = -16.5$, (*c* 1, MeOH); ^{1}H NMR for aglycon (500 MHz): Me-23 (δ 1.12); Me-24 (δ 0.86); Me-25 (δ 0.99); Me-26 (δ 1.21); Me-27 (δ 1.33); Me-29 (δ 1.11); H_a-28 (δ 3.00, d, J = 12 Hz), H_b-28 (δ 3.52, d, J = 12 Hz), H-16 (δ 3.97, br s) H-3 (δ 3.17, J = 10 and 4.5 Hz), H-30 (δ 9.5, s); ^{13}C NMR for aglycon, see literature;[2] FABMS m/z 1205 [M − H]$^-$.

Compound 5: $[\alpha]^{25}_D = -22.0$, (*c* 1, MeOH); ^{1}H and ^{13}C NMR, see literature.[20−21]

Compound 6: $[\alpha]^{25}_D = -37.2$, (*c* 1, MeOH); ^{1}H and ^{13}C NMR, see literature.[20−21]

Compound 7: $[\alpha]^{25}_D = -62.7$, (*c* 1, MeOH); ^{1}H and ^{13}C NMR, see literature.[20−21] The structures of **5**−**7** were assigned by comparison of NMR data with those reported in the literature.[21]

Antiviral Assays. The anti-HIV activity and toxicity of compounds were assessed in C8166 cells infected with HIV-1_{MN}. The cells were cultured in RPMI 1640 with 10% fetal calf serum. Forty-thousand cells per microtiter plate well were mixed with 5-fold dilutions of compounds prior to addition of 10 $CCID_{50}$ units of virus and incubated for 5−6 days. Formation of syncitia was examined from 2 days post-infection. The inhibition of HIV-infection was assessed by examining syncytia, by estimating antigen gp120 by ELISA,[23] and by measuring cell viability of virus-infected cells and uninfected cell controls using the XTT-formazan method.[24]

Virus Infectivity Assay. The total progeny virus was titrated in microtiter plates using double dilutions of freshly collected supernatants and C8166 cells. The end point was determined by examining syncytia formation and by the XTT-formazan method. The virus titer ($CCID_{50}$) is expressed as the reciprocal of the dilution that gave a 50% end point. To measure the effects of compounds on virus infectivity, HIV-1_{IIIB} (10^4−10^5 $CCID_{50}$) was incubated with test compound at 37 °C for 1 h, the virus was serially diluted, and the infectivity end-point determined. In each case the compound was diluted to well below the EC_{50} such that residual compound did not interfere with the virus titration.

Gp120-sCD4 and gp120-Antibody-Binding Assays. Gp120-sCD4 interaction was measured by ELISA, sCD4 was bound to microtiter plate wells at a concentration of 0.05 μg/well. Various dilutions of compounds were mixed with equal volumes of recombinant gp120 (0.04 μg/mL) and added to CD4 coated wells. After incubation at 37 °C for 3−5 h, the binding of gp120 was detected using human anti-HIV serum and anti-human Ig conjugated to horseradish peroxidase. Using WIA-CALC (Pharmacia LKB) the percent inhibition was calculated from linear logarithmic plots using three concentrations of gp120 alone as standard. Binding of anti-gp120 monoclonal antibodies ADP323, ADP358, and ADP360 to gp120 immobilized on microtiter plate wells was assayed in a similar manner, in the presence of various concentrations of compounds.

Acknowledgment. This research was supported by 40% and 60% grants from the Ministero della Ricerca Scientifica e Tecnologica (MURST), Italy. The NMR was performed at the Centro di Ricerca Interdipartimentale di Analisi Strumentale and FABMS spectra were performed at the Servizio di Spettrometria di Massa of the University "Federico II," Naples. The assistance of the staff is gratefully appreciated.

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NP960074H