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New metabolites with antibacterial activity from the marine angiosperm *Cymodocea nodosa*

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Abstract

Four new metabolites (**1**–**4**) have been isolated from the organic extract of the seagrass *Cymodocea nodosa*, collected at the coastal area of Porto Germeno, in Attica Greece. Compounds **1** and **2** belong to the structural class of diarylheptanoids, which have been found only once before in marine organisms [Kontiza, I.; Vagias, C.; Jakupovic, J.; Moreau, D.; Roussakis, C.; Roussis, V. *Tetrahedron Lett.* **2005**, *46*, 2845–2847]. Compound **3** is a new meroterpenoid, while compound **4**, to the best of our knowledge, is the first briarane diterpene isolated from seaweeds, and only the second analog of this class with a tricyclic skeleton. Furthermore metabolite **4** is the first brominated briarane diterpene. The structures and the relative stereochemistry of the new natural products were established by spectral data analyses. The new metabolites were submitted for evaluation of their antibacterial activity against multidrug-resistant (MDR) pathogens including methicillin-resistant (MRSA) strains of *Staphylococcus aureus*, as well as the rapidly growing mycobacteria, *Mycobacterium phlei*, *Mycobacterium smegmatis*, and *Mycobacterium fortuitum*.

Keywords: *Cymodocea nodosa*; Diarylheptanoids; Meroterpenoid; Briarane diterpene; Antibacterial activity

1. Introduction

The marine angiosperm *Cymodocea nodosa* (Ucria) Ascherson, significantly influences the local ecosystem by amplifying the primary substrate and by providing a spatially diverse nursery habitat structure and resources for algal and animal communities, many of which are commercially important.[2–4] *C. nodosa* is one of the six species in the Mediterranean Sea that are recognized as seagrasses. The seagrasses' contribution to global marine primary productivity and their role as 'structural' species in the marine biodiversity of coastal environments, have led to the inclusion of their habitat for protected ecosystems in the UNCED Action Paper (Agenda 21).

Despite the important ecological role of *C. nodosa* in the marine ecosystem, knowledge of its chemical content is limited. In particular, molecules frequently found in terrestrial plants such as caffeic acid, inositol, sucrose, monoglucoside of quercetin, monoglucoside of isoramnetin, cichoric acid, as well as polyamines like putrescine, spermidine, and spermine, have been reported as constituents of *C. nodosa* in the literature.[5–7] Furthermore, 24α-ethyl sterols and 24α-methyl sterols along with their 24β-epimers, cymodiene and cymodienol, the first diarylheptanoids isolated from marine organisms, comprise the total number of metabolites isolated from *C. nodosa*.[1,8] It is believed that *C. nodosa* originated from terrestrial ancestors and returned to the sea,[9,10] during the period of the ancient Tethys Sea, surrounded by Africa, Gondwanaland, and Asia, approximately 100 million years ago, thus explaining the 'terrestrial-like' chemical profile of the seagrass.

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In the course of our ongoing research activities toward the isolation of bioactive metabolites from marine organisms from the Greek seas,[11,12] we were recently able to collect and analyze specimens of *C. nodosa* from the sandy marine plains of Porto Germeno near Athens. Herein, we report the isolation, structure elucidation, and antibacterial properties of two biphenyl compounds deoxycymodienol (**1**) and isocymodiene (**2**), the meroterpenoid nodosol (**3**), and the brominated briarane diterpene (1*S**,2*S**,3*S**,7*R**,8*S**,9*R**,11*R**,12*S**,14*R**)-7-bromo-tetradecahydro-12-hydroxy-1-isopropyl-8,12-dimethyl-4-methylenephenanthren-9,14-yl diacetate (**4**).

2. Results and discussion

C. nodosa specimens were collected and freeze dried during June of 2005 from the coastal area of Porto Germeno, and subsequently extracted exhaustively with a mixture of dichloromethane/methanol (3:1) to afford a brownish oily extract (27.1 g) that was subjected to fractionation with a combination of chromatographic techniques, such as vacuum liquid chromatography on silica gel (VLC), solid phase extraction (SPE), preparative TLC, and normal phase HPLC, to allow the isolation of (**1**) (2.3 mg), (**2**) (3.8 mg), (**3**) (2.3 mg), and (**4**) (1.2 mg) in pure form.

Compound **1** was isolated as a colorless oil. The $[M]^+$ ion at *m/z* 278.1298 observed in the high-resolution (HR) mass spectrum in combination with the ^{13}C NMR data indicated a molecular formula of $C_{19}H_{18}O_2$. The IR and UV bands at 1635 cm^{-1} and 295 nm, respectively, were characteristic for the presence of a benzene ring.[13,14] The presence of 16 aromatic/olefinic carbons in the ^{13}C NMR spectrum in combination with 11 degrees of unsaturation suggested a tricyclic structure. The 1D ^{13}C and DEPT NMR spectra revealed the presence of 3 methylene, 10 methine, and 6 quaternary carbons. Two deshielded carbons resonating at δ 150.0 and 150.9 ppm implied that they were each attached to an electronegative atom, such as oxygen. The doubly allylic protons at δ 3.50/3.25 ppm were coupled with the first proton of a diene system at δ 5.95 ppm. The spin system of the conjugated double bonds was clearly resolved by the $^1H-^1H$ COSY experiment, additionally showing the coupling of an allylic methylene (δ 2.88 and 2.40 ppm) with a second benzylic methylene (δ 2.60 and 2.88 ppm). The *E* and *Z* geometry of the double bonds is proposed on the basis of the coupling constants ($J_{2,3}$=15.5 Hz, $J_{4,5}$=11.0 Hz). Analysis of the 2D-heteronuclear experiments revealed the biphenyl system of two ABX aromatic spin systems. The observed long range heteronuclear correlations of H-1b with C-2′ and H-9, H-13 with C-7 (Table 1), the chemical shifts of the aromatic carbons, and the spectral similarities with metabolites alnusdiol and cymodienol (**5**) previously isolated from the tree *Alnus japonica* and from the seagrass *C. nodosa*, respectively,[15,1] confirmed the arrangement of the trisubstituted aromatic rings. On the basis of the above-mentioned data, along with conformational analysis performed using the Monte Carlo method and OPLS-AA force,[16,17] and the calculated ^1H and ^{13}C NMR shielding

Table 1
NMR data for deoxycymodienol (**1**) and isocymodiene (**2**)

Position	Deoxycymodienol (**1**)			Isocymodiene (**2**)			δ_H,[b] m, *J* (Hz)
	δ_H,[a] m, *J* (Hz)	δ_C[a]	HMBC[a]	δ_H,[a] m, *J* (Hz)	δ_C[a]	HMBC[a]	
1a	3.50, m	32.5	C-3	5.15, dd, 17.3, 1.6	115.6	C-2, C-3	5.12, dd, 17.3, 1.9
1b	3.25, m	32.5	C-2′	5.12, dd, 10.4, 1.6		C-2, C-3	5.01, dd, 10.4, 1.9
2	5.95, ddd, 7.9, 7.9, 15.5	136.5	C-1, C-3	5.97, ddd, 17.3, 10.4, 5.7	139.3	C-3	5.96, ddd, 17.3, 10.4, 5.7
3	6.78, dd, 15.5, 11.0	128.8	C-2	4.43, dd, 8.2, 5.7	42.1	C-1, C-2, C-4′, C-3′	4.65, dd, 8.2, 5.7
4	6.05, dd, 11.0, 10.7	128.1		5.53, dd, 10.7, 8.2	129.5	C-3, C-6	5.60, dd, 9.9, 8.2
5	5.40, m	131.8		5.60, ddd, 10.7, 7.6, 6.6	131.3	C-3	5.50, m
6a	2.88, m	28.2	C-7, C-5	2.40, ddd, 14.8, 7.6, 6.6	29.6	C-7, C-8, C-5, C-4	2.36, m
6b	2.40, m	28.2	C-7, C-5				
7a	2.60, m	31.7	C-6, C-8, C-5	2.58, m	34.7	C-6, C-8, C-13, C-9	2.48, m
7b	2.88, m	31.7	C-6, C-8, C-5				
8	—	129.7	—	—	133.9	—	—
9	7.21, d, 2.2	135.1	C-7, C-11, C-13	7.00, d, 8.5	129.5	C-7, C-13, C-11, C-10	6.89, d, 8.5
10	—	126.4	—	6.71, d, 8.5	115.2	C-8, C-11, C-12	6.71, d, 8.5
11	—	150.0	—	—	153.7	—	—
12	6.74, d, 8.1	114.7	C-10, C-11	6.71, d, 8.5	115.2	C-8, C-10, C-11	6.71, d, 8.5
13	6.97, dd, 2.2, 8.1	129.2	C-9, C-11, C-7	7.00, d, 8.5	129.5	C-7, C-9, C-12, C-11	6.89, d, 8.5
1′	—	133.8	—	—	154.0	—	—
2′	7.60, d, 2.5	139.7	C-1, C-3′, C-4′, C-6′	6.65, d, 0.9	114.6	C-4′, C-3, C-6′	6.88, d, 3.0
3′	—	126.2	—	—	130.1	—	—
4′	—	150.9	—	—	147.6	—	—
5′	6.63, d, 8.0	114.1	C-3′, C-4′	6.72 overlapped	117.1	C-3′, C-4′	6.50, d, 8.6
6′	7.04, dd, 2.5, 8.0	128.2	C-4′, C-2′	6.66 overlapped	112.4	C-4′, C-2′	6.57, dd, 8.6, 3.0
OH-C-4′	n.d.	—		n.d.	—		n.d.
OH-C-11	n.d.	—		4.67, br s	—	—	n.d.
OMe-C-1′	—	—		3.73, s	55.7	C-1′	3.36, s

[a] Measured in $CDCl_3$.
[b] Measured in C_6D_6, n.d.: not detected.

constants by pseudospectral methods[18] the structure of the (**1**) is diarylheptanoid deoxycymodienol proposed as shown in Figure 1. The calculated chemical shifts are: (a) for ^{1}H NMR: δ (ppm) 3.51 (H-1a), 2.90 (H-1b), 6.19 (H-2), 7.00 (H-3), 6.15 (H-4), 5.61 (H-5), 2.81 (H-6a), 2.51 (H-6b), 2.61 (H-7a), 2.91 (H-7b), 7.58 (H-9), 6.93 (H-12), 6.92 (H-13), 7.61 (H-2′), 6.72 (H-5′), 7.04 (H-6′); ^{13}C NMR: δ (ppm) 39.1 (C-1), 136.8 (C-2), 122.8 (C-3), 125.3 (C-4), 127.5 (C-5), 31.8 (C-6), 34.7 (C-7), 134.2 (C-8), 116.1 (C-9), 126.0 (C-10), 143.8 (C-11), 107.1 (C-12), 123.0 (C-13), 126.8 (C-1′), 135.2 (C-2′), 123.7 (C-3′), 149.2 (C-4′), 111.7 (C-5′), 124.4 (C-6).

Metabolite **2** was obtained as a colorless oil. The molecular formula deduced from the HRESIMS showing *m/z* 309.1485 ([M−H]$^-$) and ^{13}C NMR data was $C_{20}H_{22}O_3$. The intense absorbances in the IR spectrum at 3375 (broad band), 2922, and 1514 cm^{-1}, indicated the presence of an hydroxyl group, an *exo*-methylene double bond, and a benzene ring, respectively. Additionally, UV absorbances at 239 and 285 nm were indicative of the presence of a phenolic ring.[19] Seven overlapping aromatic protons along with five olefinic protons were observed in the ^{1}H NMR spectrum measured in $CDCl_3$. The ^{13}C NMR spectrum in combination with the DEPT spectrum revealed the presence of 5 quaternary, 11 tertiary, 3 secondary (one sp^2 and two sp^3), and 1 primary carbon. Additionally three quaternary carbons at δ 153.7, 154.0, and 147.6 ppm along with the primary carbon at δ 55.7 ppm, indicated the presence of phenolic rings and a methyl group. The significant spectral data similarities between metabolite **2** and the previously reported diarylheptanoid cymodiene,[1] drove us to the conclusion that they bear similar structures. When the ^{1}H NMR spectrum of **2** was recorded in C_6D_6, the shielding of aromatic protons led to a much better resolved set of peaks in the aromatic region. Two doublets at δ 6.89 and 6.71 (J=8.5 Hz) ppm each integrating for two protons, indicated a *para* substituted phenyl ring. The remaining aromatic signals required a 1,2,4-trisubstituted second aromatic ring. The spin system of the seven carbon chain linking the phenolic rings was clarified by the ^{1}H−^{1}H COSY correlations. The *Z* configuration of the second double bond (Δ_4) was deduced from the coupling constant of $J_{4,5}$=10.7 Hz. The heteronuclear correlation of the benzylic carbon at δ 34.7 ppm with the aromatic proton at δ 7.00 ppm secured the position of the disubstituted phenyl ring of metabolite **2**, and this new natural product is assigned the trivial name isocymodiene. The strong NOE effects (in C_6D_6) between the methoxy singlet and the *meta* coupled aromatic protons at δ 6.88 (d) and 6.57 (dd) ppm (Fig. 2), indicated the positions of the hydroxy and methoxy moieties on the second aromatic ring. All connectivities as presented above were confirmed by the HMBC data and are included in Table 1.

The HRFABMS ([M]$^+$ 228.1528) of metabolite **3**, a colorless oil, suggested a molecular formula of $C_{16}H_{20}O$. The ^{13}C NMR spectrum of **3** in $CDCl_3$ displayed 16 signals, including 12 signals in the aromatic/olefinic region. The resonances in the ^{1}H NMR spectrum at δ_H 4.79 (1H, br s), 5.09 (1H, br s), 5.03 (1H, d, J=17.5 Hz), and 4.98 (1H, d, J=10.6 Hz) ppm, in combination with the presence of two sp^2 secondary carbons at δ_C 110.8 and 115.8 ppm, as DEPT experiments indicated, confirmed the presence of two *exo*-methylene groups. The IR bands at 3400 and 1609 cm^{-1} suggested a phenolic ring, a fact that was also supported by the presence of an exchangeable signal at δ_H 4.59 ppm (br s). Two doublets at δ 6.70 (2H, d, J=8.8 Hz) and 7.33 (2H, d, J=8.8 Hz) ppm, indicated a *para* substituted phenyl ring. The seven degrees of unsaturation, in combination with the presence of a phenolic ring, two *exo*-methylene groups, and one trisubstituted double bond at δ_H 6.20 ppm (1H, s) and δ_C 122.7 ppm suggested a monocyclic skeleton for compound **3**. This evidence along with the existing literature,[20] drove us to the conclusion that metabolite **3** was a meroterpenoid. HMBC correlations between the chemically equivalent methyl groups at δ_H 1.22 ppm and the tertiary carbons at δ_C 42.8 and 149.0 ppm, fixed their position at the side chain. Furthermore the vinyl methyl at δ_H 1.77 ppm (3H, s) showed strong correlations with the tertiary carbons at δ_C 144.1 and 149.0 ppm, as well as with the secondary sp^2 carbon at δ_C 115.8 ppm, facts that secured the carbon sequence of the side chain. Meanwhile the strong NOE effects of the olefinic proton at δ_H 6.20 ppm and the vinyl methyl at δ_H 1.77 ppm with the aromatic proton at δ_H 7.33 ppm (Fig. 2), confirmed their proximity to the aromatic ring. Finally the *para* position of the hydroxyl group on the aromatic ring was clarified by its strong NOE correlation with the aromatic protons at δ_H 6.70 ppm and the chemical equivalence of the two pairs of aromatic protons as well as their *ortho* coupling (J=8.8 Hz) (Fig. 2). The new

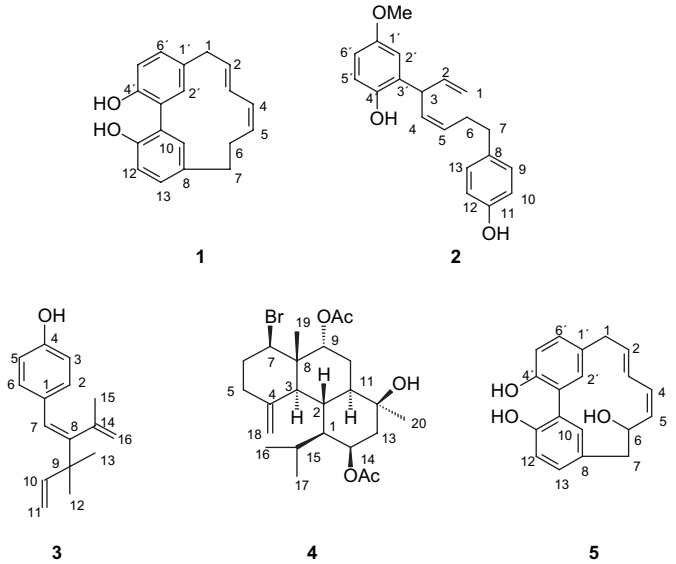

Figure 1. Metabolites isolated from *C. nodosa*.

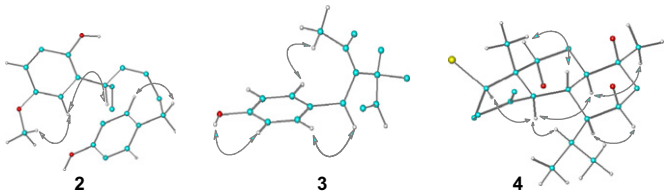

Figure 2. NOESY correlations of metabolites **2**–**4**.

Table 2
NMR data for nodosol (**3**) in $CDCl_3$

Position	δ_H, m, J (Hz)	δ_C	HMBC
1	—	130.7	
2	7.33, d, 8.8	129.9	C-4, C-6, C-3
3	6.70, d, 8.8	114.8	C-4, C-5, C-1
4	—	154.0	
5	6.70, d, 8.8	114.8	C-4, C-3, C-1
6	7.33, d, 8.8	129.9	C-4, C-2, C-5
7	6.20, s	122.7	C-6, C-2, C-8, C-14, C-9
8	—	149.0	
9	—	42.8	
10	5.91, dd, 17.5, 10.6	147.6	C-9, C-12, C-13
11a	5.03, d, 17.5	110.8	C-9, C-10
11b	4.98, d, 10.6	110.8	C-9, C-10
12	1.22, s	26.5	C-9, C-13, C-10
13	1.22, s	26.5	C-9, C-12, C-10
14	—	144.1	
15	1.77, s	24.7	C-16, C-8, C-14
16a	5.09, br s	115.8	C-8, C-9, C-15
16b	4.79, br s	115.8	C-8, C-9, C-15
OH-C-4	4.59, br s	—	C-4, C-3, C-5

meroterpenoid was named nodosol (**3**), and its spectral data are given in Table 2.

Metabolite **4** was purified by HPLC and was isolated as a colorless oil. Both ^{13}C NMR data and HRESIMS measurements supported the molecular formula $C_{24}H_{37}BrO_5$. The presence of one bromine atom was indicated by the EIMS exhibiting $[M-AcO]^+$ peaks at *m/z* 424 and 426 with intensities of 1:1. Furthermore the fragment at *m/z* 285, corresponding to $[M-2AcO-Br]^+$, in combination with a strong band in the IR spectrum at 1734 cm^{-1}, indicated the presence of two acetoxy groups. The ^{13}C NMR spectrum along with the DEPT experiments showed the presence of five quaternary, eight methine, five methylene, and six methyl carbon atoms. Among them, two were olefinic at δ_C 145.0 ppm (quaternary) and 109.4 ppm (secondary), suggesting the presence of an *exo*-methylene group, four of them were contributing to the two acetoxy groups (δ_C 21.2, 169.8, 21.7, and 169.6 ppm), and finally four were resonating in the chemical shift region of oxygenated/halogenated carbons (δ_C 75.6, 72.3, 71.0, and 59.0 ppm). Additionally the ^1H NMR spectrum revealed the presence of three oxygenated or halogenated methines at δ_H 5.05 (dd, J=3.8, 1.6 Hz), 5.31 (br s), and 4.37 (dd, J=12.1, 4.9 Hz) ppm, two secondary methyl groups at δ_H 0.58 (d, J=6.8 Hz) and 0.89 (d, J=6.8 Hz) ppm forming an isopropyl group and two methyls at δ_H 1.05 (s) and 0.85 (s) ppm situated on quaternary carbons. The presence of two acetoxy groups was also confirmed by two singlets at δ_H 2.11 and 2.06 ppm, each of them integrating for three protons, while the two broad singlets at δ_H 4.95 (1H) and 4.76 (1H) ppm were associated with the *exo*-methylene group. The NMR data of this compound (Table 3) were similar to those of briarane diterpenes, which have been isolated in the past from *Pennatulacea* and *Gorgonacea* octocorals, and a Mediterranean nudibranch.[21,22]

The six degrees of unsaturation, combined with the presence of one *exo*-methylene group and two acetoxy groups, suggested that compound **4** should be tricyclic. The HMBC

Table 3
NMR data for compound **4** in $CDCl_3$

Position	δ_H, m, J (Hz)	δ_C	HMBC	NOESY
1α	1.24, m	55.6	C-2, C-3, C-16, C-11	H-13α, H-3α, H-14α
2β	2.27, m	31.2	C-3, C-1	H-16, H-19
3α	2.00, m	47.7	C-7, C-1, C-18, C-4	H-11α, H-7α, H-1α, H-6α
4	—	145.0		
5α	2.11, m	35.7	C-3, C-7, C-18, C-4	
5β	2.29, m		C-3, C-7, C-4	H-19, H-6β
6α	2.34, m	38.3	C-7, C-5, C-8, C-18, C-4	H-3α
6β	2.08, m		C-5	H-5β
7α	4.37, dd, 12.1, 4.9	59.0		H-3α, H-6α, H-6β
8	—	44.2		
9β	5.05, dd, 3.8, 1.6	75.6	C-11	H-10β, H-19
10α	1.92, ddt, 14.8, 3.8, 3.8	23.5		H-11α
10β	1.74, ddd, 14.8, 14.8, 1.6		C-12	H-9β
11α	1.37, m	47.6		H-1α, H-3α, H-20, H-10α
12	—	72.3		
13α	1.60, dd, 14.9, 3.5	44.9	C-20	H-20
13β	2.01, m		C-14, C-1	H-20
14α	5.31, br s	71.0	C-12, C-2	H-13α, H-1α, H-13β, H-16, H-17
15	1.80, m	27.1	C-14, C-1	
16	0.58, d, 6.8	18.7	C-1, C-17	H-2β, H-18a, H-14α
17	0.89, d, 6.8	25.2	C-1, C-15, C-16	H-2β, H-14α
18a	4.76, s	109.4	C-3, C-4, C-6	H-2β, H-19, H-16
18b	4.95, s		C-3, C-6	H-5β
19	0.85, s	16.1	C-8, C-9, C-3, C-7	H-9β, H-10β, H-2β
20	1.05, s	27.8	C-11, C-12, C-13	H-11α, H-13α
MeCOO on C-14	2.06, s	21.7	C=O (C-14)	H-17
MeCOO on C-14	—	169.6		
MeCOO on C-9	2.11, s	21.2	C=O (C-9)	H-7α
MeCOO on C-9	—	169.8		

experiment revealed correlations between H_3-19 and C-8, C-3, C-7, and C-9, confirming the ring fusion between C-8 and C-3 as expected in briarane diterpenes. However, the cross peaks between H-11 and C-1 observed in the HMBC spectrum (see Table 3), in combination with the correlation between H-2 and H-11, which was shown in the $^1H-^1H$ COSY spectrum, along with the multiplicities of C-2 and C-11, suggested a bond between these carbons. This is the second naturally occurring briarane diterpene possessing such a ring fusion and the first case of a brominated briarane.[23] The relative stereochemistry for compound **4** was determined on the basis of the NOESY spectrum and $^1H-^1H$-coupling constants. In particular NOE enhancements between H_3-19 and H-9 possessing an equatorial configuration as its coupling constant (J=3.8, 1.6 Hz) suggested, confirmed their cis (β) configuration. Furthermore, observed NOE effects between H-3α and H-11α, H-7α, H-1α along with the NOE interactions between H-11α/H_3-20, H-1α/H-14α and H-14α/H-13α proved their cis (α) configuration and confirmed the trans-fusion of the rings at C-8/C-3 and C-11/C-2 (Fig. 2). Compound **4** is a new briarane diterpene named (1*S**,2*S**,3*S**,7*R**,8*S**,9*R**,11*R**,12*S**,14*R**)-7-bromo-tetradecahydro-12-hydroxy-1-isopropyl-8,12-dimethyl-4-methylenephenanthren-9,14-yl diacetate (**4**).

Metabolites (**1**–**5**) isolated from *C. nodosa* were assayed for evaluation of their antibacterial activity against multidrug-resistant (MDR) and methicillin-resistant strains of *Staphylococcus aureus* (MRSA) as well as rapidly growing mycobacteria, including *Mycobacterium phlei*, *Mycobacterium smegmatis*, and *Mycobacterium fortuitum*, which are used as an alternative screening model to *Mycobacterium tuberculosis* for evaluation of antitubercular drugs.[24] These species have obvious advantages over *M. tuberculosis* since they can be handled in class 2 microbiological laboratories and are fast growing strains, with a completion time for one assay of 72 h. The observed activity ranged from weak to strongly active. In general compounds **1**–**3** were found to be more active against MDR strains than against the standard *S. aureus* strain ATCC 25923. Specifically nodosol (**3**) was the most active compound in the assays (Table 4) leading to a threefold lower minimum inhibitory concentration (MIC) in comparison with that of tetracycline against the effluxing strain XU212 possessing the Tetk pump. A twofold lower MIC compared to that of the fluoroquinolone norfloxacin was observed for nodosol (**3**) against strain SA1199B possessing the NorA MDR efflux transporter, which is resistant to certain fluoroquinolones. The same metabolite (**3**) against RN4220, which possesses the MsrA macrolide efflux protein and is resistant to erythromycin, exhibited a fourfold lower MIC compared to that of erythromycin. The meroterpenoid bakuchiol isolated from the seeds of *Psoralea corylifolia*,[25] which shows similarities in its structure with nodosol (**3**), has exhibited in the past strong activity against different strains of *Streptococcus*.[26] During this study we had the opportunity to re-isolate the diarylheptanoid cymodienol (**5**), which has previously been shown to be cytotoxic against cancer cell lines A549 and NSCLC-N6-L16[1] and we have evaluated its antibacterial activity (Table 4). Deoxycymodienol (**1**) was slightly more active compared to cymodienol (**5**) against all examined strains. Isocymodiene (**2**) exhibited strong to moderate activity against MDR and MRSA strains of *S. aureus*. Compound **4** did not show any inhibition against any of the examined strains of *S. aureus*. In view of the constantly increasing number of fatal incidences attributed to MRSA[27] and the reported resistance to vancomycin and linezolid in MRSA,[28,29] further investigations in the diarylheptanoid and meroterpenoid chemical classes as antistaphylococcal leads should be continued.

Among the compounds tested, nodosol (**3**) displayed the most potent inhibitory activity with MIC value of 16 μg/ml against *M. fortuitum*, *M. phlei*, and *M. smegmatis* (Table 5). Cymodienol (**5**) has an additional hydroxyl group compared to metabolite **1** and showed no activity against *M. fortuitum*, which in comparison with the observed MIC (64 μg/ml) of deoxycymodienol (**1**) against the same strain, supports the hypothesis that polarity seems to influence the in vitro antimycobacterial activity previously reported in the literature.[30,31] Higher lipophilicity may hold a key role in the antimycobacterial activity, due to the lipophilic nature of the mycobacterial cell wall. In contrast to this against *M. phlei*, deoxycymodienol (**1**) exhibited a fourfold higher MIC in comparison to cymodienol (**5**). Isocymodiene (**2**) showed moderate activity against *M. fortuitum* and *M. phlei* with an MIC of 32 μg/ml. To the best of our knowledge this is the first report of antimycobacterial activity of diarylheptanoids. The simple structures of the meroterpenoid nodosol (**3**) and the diarylheptanoids, cymodienol (**5**), deoxycymodienol (**1**), and isocymodiene (**2**), make them feasible targets for the synthesis and chemical

Table 4
Minimum inhibitory concentrations (MIC)[a] of metabolites **1**–**5** against strains of *S. aureus*

Metabolite	Strain (resistance mechanism)					
	SA1199B (NorA)	RN4220 (MsrA)	XU212 (TetK, mecA)	ATCC 25923	EMRSA-15	CD-1281 (TetK)
1	32	32	32	32	n.t.	n.t.
5	64	64	64	64	64	64
2	32	64	64	64	n.t.	n.t.
3	16	16	16	16	16	16
4	>128	>128	>128	n.t.	n.t.	n.t.
Norfloxacin	32			0.5	0.5	
Erythromycin		128				
Tetracyclin			128			32

[a] All MICs (μg/ml) were determined in duplicate, n.t.: not tested.

Table 5
Minimum inhibitory concentrations (MIC)[a] of metabolites **1**–**5** against fast growing species of *Mycobacterium*

Metabolite	Strain		
	M. smegmatis	*M. fortuitum*	*M. phlei*
1	n.t.	64	64
5	128	>128	16
2	n.t.	32	32
3	16	16	16
4	n.t.	>128	>128
Ethambutol	0.5	8	2

[a] All MICs (μg/ml) were determined in duplicate, n.t.: not tested.

modification that can lead to optimization of their antibacterial activity.

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured using a Perkin–Elmer model 341 polarimeter and a 10 cm cell. UV spectra were determined in spectroscopic grade CH_2Cl_2 and $CHCl_3$ on a Shimadzu UV-160A spectrophotometer. IR spectra were obtained using a Paragon 500 Perkin–Elmer spectrophotometer. NMR spectra were recorded using a Bruker AC 200, Bruker DRX 400, and Bruker Avance 500 MHz spectrometers. Chemical shifts are given on a δ (ppm) scale using TMS as internal standard. The 2D experiments ($^1H-^1H$ COSY, HMQC, HMBC, and NOESY) were performed using standard Bruker microprograms. High-resolution mass spectral data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, Indiana, USA. EIMS data were recorded on a Hewlett Packard 5973 Mass Selective Detector. VCC separation was performed with Kieselgel 60H (Merck), TLC was performed with Kieselgel $60F_{254}$ aluminum support plates (Merck) and spots were detected with 15% H_2SO_4 in MeOH reagent. HPLC separation was conducted using a Pharmacia LKB 2248 model equipped with a refractive index detector RI GBC LC-1240 and a Spherisorb HPLC normal phase column, 25 cm×10 mm, S10W, 64,340 plates/meter.

3.2. Plant material

The seagrass was collected by hand at Porto Germeno in Corinthiakos Gulf in Greece, at a depth of 0.5–1 m during the summer of 2005. A voucher specimen is kept at the Herbarium of the Pharmacognosy Laboratory, University of Athens.

3.3. Extraction and isolation

The organism was initially freeze dried (1.16 kg dry weight) and then exhaustively extracted at room temperature with mixtures of CH_2Cl_2/MeOH (3:1). The organic extract, after vacuum evaporation of the solvents, afforded a brownish oily residue (27.1 g). The crude extract was subjected to VLC on silica gel using cyclohexane with increasing amounts of EtOAc and finally MeOH as eluants. Fractions eluted with 20% (A), 22% (B), 24% (C), and 32% (D) of EtOAc in cyclohexane were further purified. Fraction C was subjected to VLC on silica gel starting from 100% cyclohexane with increasing amounts of EtOAc. The fraction eluted with 50% EtOAc was further purified with reverse phase SPE, Phenomenex Strata silica (10 g/60 ml giga tubes) using a step gradient system from 100% H_2O to 100% MeOH, yielding 11 fractions. The fraction eluted with 100% MeOH contained compound **1** in pure form (2.3 mg). Fraction D was further purified by HPLC (column Spherisorb; S10W 25 cm×10 mm), using as mobile phase of cyclohexane/EtOAc (85:15) to yield pure compound **2** (3.8 mg), which had a retention time of 37.1 min (flow rate 2 ml/min). Fraction B was further purified with SPE, using a reverse-phase column (Phenomenex Strata silica, 10 g/60 ml giga tubes) starting from 100% H_2O with increasing amounts of MeOH. The fraction eluted with 100% MeOH was subjected to preparative TLC, using as mobile phase of cyclohexane/EtOAc (80:20) to finally afford compound **3** (2.3 mg) (R_f: 0.30). Finally fraction A was subjected again to VLC on silica gel using cyclohexane with increasing amounts of EtOAc. The fraction eluted with 30% EtOAc (A1) was subjected to normal phase HPLC, column Spherisorb; S10W 25 cm×10 mm), using as mobile phase cyclohexane/EtOAc (78:22), to yield pure compound **4** (1.2 mg), which had a retention time of 29.0 min (flow rate 2 ml/min).

3.3.1. Compound *1*

Colorless oil; UV (CH_2Cl_2) λ_{max} (log ε) 254.0 (0.39), 295.0 (0.10); IR (film) ν_{max} 3354, 2930, 1716, 1635, 1576, 1507, 1235 cm^{-1}; HREIMS: *m/z* $[M]^+$; 278.1298 ($C_{19}H_{18}O_2$, calcd 278.1302); NMR data (500 MHz; $CDCl_3$), see Table 1.

3.3.2. Compound *2*

Colorless oil; $[\alpha]_D^{25}$ +0.35 (*c* 0.06, CH_2Cl_2); UV ($CHCl_3$) λ_{max} (log ε) 285.0 (0.84), 239.0 (0.97), 229.0 (0.61); IR (film) ν_{max} 3375, 2922, 2851, 1716, 1643, 1514, 1202 cm^{-1}; HRESIMS: *m/z* $[M-H]^-$; 309.1485 ($C_{20}H_{21}O_3$, calcd 309.1496); NMR data (500 MHz; $CDCl_3$ and 500 MHz; C_6D_6) see Table 1.

3.3.3. Compound *3*

Colorless oil; UV (CH_2Cl_2) λ_{max} (log ε) 250 (3.7), 260.5 (3.8), 272.5 (2.8), 295.0 (2.7); IR (film) ν_{max} 3400, 2965, 1609, 1509 cm^{-1}; HRFABMS *m/z* 228.1528 $[M]^+$ ($C_{16}H_{20}O$, calcd 228.1514); NMR data (500 MHz; $CDCl_3$), see Table 2.

3.3.4. Compound *4*

Colorless oil; $[\alpha]_D^{25}$ +3.5 (*c* 0.06 CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 233 (2.2), 218 (2.7); IR (film) ν_{max} 2927, 1734 cm^{-1}; HRESIMS *m/z* 502.2163 $[M+NH_4]^+$ ($C_{24}H_{41}^{79}BrNO_5$, calcd 502.2155); NMR data (400 MHz; $CDCl_3$), see Table 3; EIMS 70 eV, *m/z* (rel int.): 424 $[M-AcO]^+$ (10), 366 (15), 285 $[M-2AcO-Br]^+$ (90), 267 $[M-2AcO-Br-H_2O]^+$ (60), 43 (100).

3.4. Bacterial strains

Ethambutol was used as a positive control for the mycobacterial strains, while for *S. aureus* strains the control antibiotics

norflorxacin, tetracycline, and erythromycin were employed. All antibiotics were obtained from the Sigma Chemical Co., *S. aureus* standard strain ATCC 25923 and tetracycline-resistant strain XU212, which is also an MRSA strain were provided by Dr. Edet Udo.[32] Strain SA1199B was provided by Professor Glenn Kaatz.[33] Strain RN4220 was provided by Dr. J. Cove.[34] Epidemic MRSA strain EMRSA-15, which accounts for the majority of MRSA bacteraemia in UK hospitals,[35] was obtained from Dr Paul Stapleton (ULSOP). Finally strain CD-1281 was generously provided as gift from Professor C. Dowson (University of Warwick, UK). Mycobacterial species were acquired from the NCTC.

3.5. Determination of MIC

S. aureus strains were cultured on nutrient agar and incubated for 24 h at 37 °C prior to MIC determination, while *Mycobacterium* strains were grown on Columbia blood agar (Oxoid) supplemented with 7% defibrinated Horse blood (Oxoid) and incubated for 72 h at 37 °C prior to MIC determination. Bacterial inocula equivalent to the 0.5 McFarland turbidity standard were prepared in normal saline and diluted to give a final inoculum density of 5×10^5 cfu/ml. Using Nunc 96-well microtitre plates, 125 μl of Mueller–Hinton broth (MHB) (Oxoid) were dispensed into wells 1–11. Test compound (125 μl) or the appropriate antibiotic were dispensed into well 1 and serially diluted across the plate leaving well 11 empty for the growth control. The final volume was dispensed into well 12, which being free of MHB or inoculum served as the sterile control. The inoculum (125 μl) was added to all wells and the microtitre plate was incubated at 37 °C for 72 h for *M. fortuitum*, *M. smegmatis*, and *M. phlei* and for 18 h for *S. aureus* strains. The MIC was recorded as the lowest concentration at which no bacterial growth was observed and this was enhanced by the addition of 20 μg/ml methanolic solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) to each of the wells followed by incubation at 37 °C for 20 min. A blue coloration indicated growth. No growth resulted in the well remaining yellow, as previously described.[32] Mueller–Hinton broth was adjusted to contain 20 and 10 mg/l of Ca^{2+} and Mg^{2+}, respectively.

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