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Anthraquinones and Betaenone Derivatives from the Sponge-Associated Fungus *Microsphaeropsis* Species: Novel Inhibitors of Protein Kinases

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An undescribed fungus of the genus *Microsphaeropsis*, isolated from the Mediterranean sponge *Aplysina aerophoba*, produces two new betaenone derivatives (**1**, **2**) and three new 1,3,6,8-tetrahydroxyanthraquinone congeners (**5**−**7**). The structures of the compounds were established on the basis of NMR spectroscopic and mass spectrometric data and by CD spectroscopy. This is the first report wherein the ^{1}H and ^{13}C NMR data of the betaenone congeners are fully and unambiguously assigned on the basis of two-dimensional NMR spectroscopy. Furthermore, we describe the first elucidation of the absolute configuration of 1-(2′-anthraquinonyl)ethanols such as **5** and **6**, by quantum chemical calculation of their circular dichroism (CD) and comparison with experimentally measured spectra. Moreover, it was shown that compounds **1**, **5**, **6**, and **7** are inhibitors of PKC-ϵ, CDK4, and EGF receptor tyrosine kinases.

In the search for new pharmaceutical or agrochemical lead structures increasing attention is being given to marine microorganisms as sources of novel bioactive compounds.[1−5] As marine microorganisms survive under harsh environmental conditions, it can be expected that they may have evolved to biosynthesize biologically interesting and chemically diverse compounds. They often live in symbiosis with soft-bodied filter-feeding invertebrates such as sponges, and their bioactive metabolites may be interpreted as chemically mediated defense mechanisms for protecting their host organism from environmental dangers such as predation. In this context sponge-associated fungi have been previously reported to yield cytotoxic metabolites which include chlorinated sesquiterpenes,[6] alkaloids such as asperazine[7] and gliotoxin,[8] orthoquinone polyketide obionins,[9] and lactone heliascolides.[10]

Here we describe the isolation and structure elucidation of two new 10-hydroxyl derivatives of betaenone (**1**, **2**) and three new 1,3,6,8-tetrahydroxyanthraquinone congeners (**5**−**7**) from a fungus of the genus *Microsphaeropsis* (Chart 1).

Results and Discussion

The hitherto undescribed fungus of the genus *Microsphaeropsis* (Deuteromycota, mitosporic fungi) was isolated from the Mediterranean sponge *Aplysina aerophoba*. The fungus was grown in liquid malt-extract medium, and the secondary metabolites were obtained from the EtOAc−

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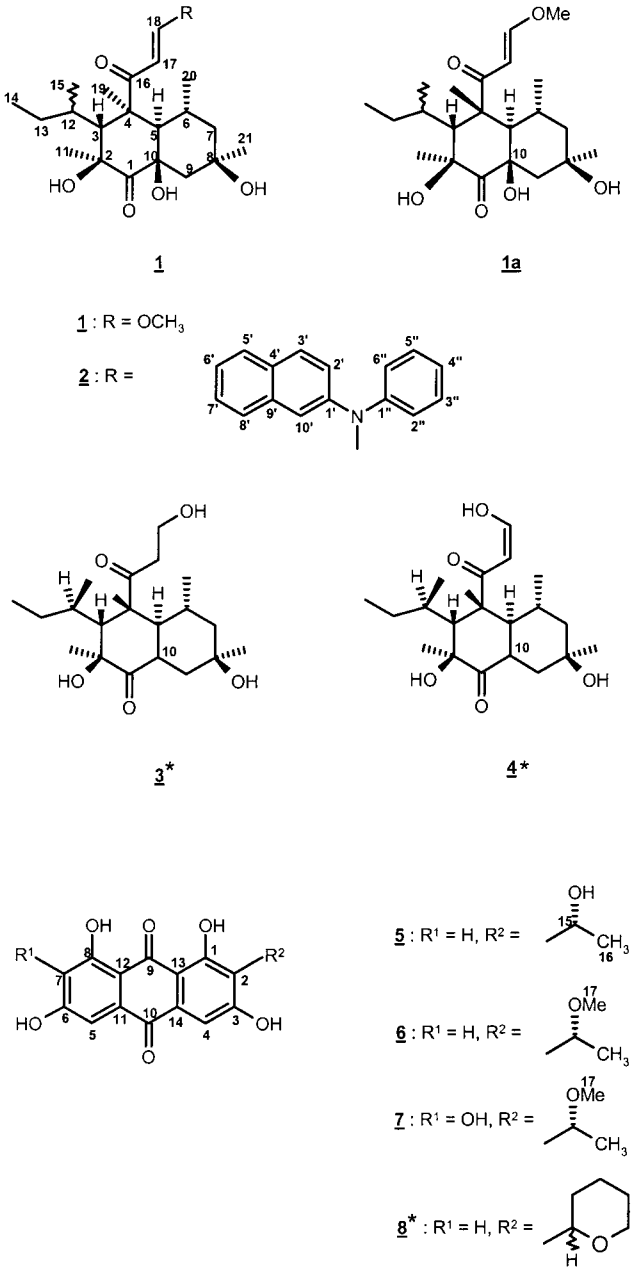
Chart 1. Isolated Compounds from the Sponge-Associated Fungus *Microsphaeropsis* sp.[a]



[a] Asterisks (*) indicate reference substances.

MeOH extract of the mycelia. Isolation of the betaenones and anthraquinones was achieved by vacuum liquid chromatography using a step gradient-elution technique employing mixtures of CH_2Cl_2 and MeOH as solvent system. The betaenones were separated from the anthraquinones by column chromatography on Sephadex LH-20 using MeOH as solvent. All compounds were readily identified from their spectroscopic data. Through-bond homonuclear ($^1H-^1H$ COSY) and heteronuclear (^1H-detected one-bond and multiple-bond ^{13}C) correlations were used to establish assignments and atom connectivities in **1**, **2**, and **5**−**7**. Chemical shifts were compared with literature data for compounds containing similar structural subunits.[11−15]

Compound **1** was obtained as a white powder. The molecular formula was determined to be $C_{22}H_{36}O_6$ (M^+ 396.2458) by HREIMS. Compound **1** is the (17*E*)-10α-hydroxy-18-*O*-methyl derivative of betaenone C, and this was confirmed from a comparison of the NMR data of **1** with those of betaenone B (**3**) and C (**4**) (Tables 1 and 2). The absolute configuration of the betaenones was deduced from an X-ray analysis, chemical correlations, and ORD/CD measurements.[16] Hence, this is the first report wherein the ^1H and ^{13}C NMR data of the betaenone congeners are fully and unambiguously assigned on the basis of two-dimensional NMR spectroscopy. Compared to betaenone C, the ^1H NMR spectrum of **1** showed an additional resonance for a methoxyl group at δ 3.83, while an additional resonance for the carbon of a tertiary hydroxyl group was found at δ 77.7 in the ^{13}C NMR spectrum. The magnitude of the vicinal coupling constant of 12.0 Hz between H-17 (δ 6.30) and H-18 (δ 7.63) indicates an *E*-configuration of the double bond in contrast to betaenone C (**4**), where a coupling constant of only 4.9 Hz defined a *Z*-configuration.[11] The assignment of the ^1H and ^{13}C signals followed from the through-bond correlations observed in the $^1H-^1H$ COSY, HMQC, and HMBC spectra, which established the atom connectivities and basic structure.

The relative configuration of **1** was determined from a ROESY experiment and the magnitudes of the coupling constants in the ^1H NMR spectrum. The chair conformation of ring B followed from the magnitude of the axial coupling constants involving H-5, H-6, and H-7 (Table 2) and the long-range W-coupling between H-7_{eq} and H-9_{eq} detected in the COSY spectrum. This was confirmed by the observation of NOEs in the ROESY spectrum from H-5 to H-7_{ax}, H-9_{ax}, Me-11, Me-19, and Me-20. The presence of a cross-

Table 1. ^{13}C NMR Data of the Betaenone Congeners **1**, **1a**, **2**, and **3**

C no.	**1**[a]	**1a**[a]	**2**[a]	**3**[b]
1	214.2 s	213.8 s	214.3 s	217.6 s*
2	79.1 s	78.9 s	79.2 s	73.3 s
3	56.6 d	56.6 d	56.6 d	57.3 d
4	53.1 s	54.3 s	52.7 s	52.9 s
5	49.3d	49.9 d	49.4 d	46.6 d
6	27.2 d	27.1 d	27.3 d	29.1 d
7	49.8 t	49.8 t	50.0 t	47.7 t
8	72.0 s	71.9 s	72.1 s	68.7 s
9	45.8 t	45.7 t	46.0 t	43.9 t
10	77.7 s	77.6 s	77.8 s	40.3 d
11	24.5 q	24.4 q	24.7 q	23.9 q
12	36.8 d	36.7 d	36.8 d	35.8 d
13	26.2 t	26.2 t	26.3 t	25.1 t
14	14.0 q	14.1 q	14.4 q	13.6 q
15	23.8 q	23.9 q	23.7 q	23.4 q
16	206.5 s	217.4 s	205.6 s	216.9 s*
17	103.7 d	103.2 d		
18	165.5 d	149.3 d		
19	21.4 q	21.8 q	21.5 q	20.4 t
20	22.5 q	22.1 q	22.3 q	21.5 t
21	30.8 q	30.8 q	30.8 q	31.1 q
22	58.8 q	58.8 q		
1′			135.4 d	
2′			142.8 s	
3′			123.5 d	
4′			131.1 d	
5′			133.0	
6′			128.9 d	
7′			127.4 d	
8′			128.2 d	
9′			128.7 d	
10′			135.4 s	
1″			145.2 s	
2″			128.2 d	
3″			131.2 d	
4″			128.1 d	
5″			131.2 d	
6″			128.2 s	

[a] Spectra were recorded in CD_3OD at 300 MHz for **1** and **1a**, and at 500 MHz for **2**. [b] Taken from ref 19 and assignments (*) are interchangeable

Table 2. ^{1}H NMR Data of the Betaenone Congeners **1**, **1a**, **2**, **3**, and **4**

H no.	**1**[a]	**1a**[a]	**2**[a]	**3**[b]	**4**[c]
3	2.02 s	2.09 s	1.86 s		
5	2.76 d 10.9 Hz	2.66 d 10.9 Hz	2.77 d 10.9 Hz	2.49 dd 14.0 Hz 11 Hz	2.58 dd 12.7 Hz 9.8 Hz
6	2.16 m	2.16 m	2.12 m		
7eq	1.76 dt 13.8 Hz 3.2 Hz	1.76 dt 13.8 Hz 3.2 Hz	1.73 dt 13.7 Hz 3.3 Hz		
7ax	1.31 dd 14.0 Hz 9.5 Hz	1.31 dd 14.0 Hz 9.5 Hz	1.27 m		
9eq	2.25 d 14.4 Hz	2.25 d 14.4 Hz	2.18 dd 14.4 Hz 2.9 Hz	2.31 dt 14.0 Hz 3.0 Hz	2.33 ddd 13.7 Hz, 3.4 Hz, 3.7 Hz
9ax	1.33 d 14.3 Hz	1.33 d 14.3 Hz	1.30 d 14.3 Hz		
10				2.66 ddd 14.0 Hz, 11 Hz, 3.0 Hz	
11-Me	1.62 s	1.57 s	1.64 s	1.57 s	1.61 s
12	1.94 m	1.94 m	1.79 m		
13	1.53 m	1.53 m	1.50 m		
14-Me	0.82 t 7.3 Hz	0.94 t 7.0 Hz	0.90 t 7.3 Hz	0.86 t 7.0 Hz	0.74 d 6.8 Hz
15-Me	1.17 d 7.0 Hz	1.21 d 7.0 Hz	1.06 d 7.0 Hz	1.15 d 6.8 Hz	1.12 d 6.8 Hz
17	6.30 d 12.0 Hz	6.30 d 12.0 Hz	5.83 d 12.7 Hz	2.82 dt 18.0 Hz 3.0 Hz	5.89 d 4.9 Hz
18	7.63 d 12.0 Hz	7.63 d 12.0 Hz	8.28 d 12.7 Hz	3.11 dq 18.0 Hz 3.0 Hz	7.74 d 4.9 Hz
19-Me	1.68 s	1.71 s	1.30 s	1.40 s	1.36 s
20-Me	0.81 d 6.5 Hz	0.80 d 6.5 Hz	0.84 d 6.6 Hz	0.67 d 6.4 Hz	0.79 t 7.3 Hz
21-Me	1.22 s	1.22 s	1.17 s	1.16 s	1.26 s
22-OCH_3	3.83 s	3.83 s			
2′			7.26 d 8.8 Hz		
3′			7.94 d 8.8 Hz		
5′			7.89 d 8.6 Hz		
6′			7.49 m		
7′			7.53 m		
8′			7.8 d 7.3 Hz		
10′			7.60 s		
2″			7.21 d 8.0 Hz		
3″			7.51 m		
4″			7.35 m		
5″			7.51 m		
6″			7.21 d 8.0 Hz		

[a] Spectra were recorded in CD_3OD at 300 MHz for **1** and **1a**, and at 500 MHz for **2**. [b] Taken from ref 19. [c] Taken from ref 20.

peak between the resonances for H-5 and Me-11 indicates a twisted conformation for ring A, as previously suggested from conformational analysis of the betaenones,[5−7] while the NOE between H-5 and Me-19 requires these to be on the same side of ring A. The *trans*-decalin configuration with an axial hydroxyl substituent is similar to that of the betaenones and stemphyloxins, which also indicates that Me-11 and Me-21 remain in an equatorial orientation. The ^{1}H and ^{13}C NMR data reveal that **1** is a mixture of two stereochemical isomers **1** and **1a** in a ratio of 2:1. It was not possible to separate the isomers. A configurational difference between the two compounds was observed at the stereogenic center C-4. For the major compound **1**, C-16 is equatorially oriented, causing a deshielding effect of 0.6 ppm on C-5, as shown in its ^{13}C NMR spectrum. However, when C-16 is at an axial orientation as in **1a**, it is deshielded by the axial hydroxyl group at C-10 of ca. 11 ppm, as found in **3** (Table 1). For the minor compound **1a**, the configurations at the stereocenters, C-3 and C-4, are identical with those of **3** and **4**, as indicated by the similarity of the NMR data to those published,[11,12] while the stereochemistry at C-12 cannot be determined.

Compound **2** was obtained as a white powder. The molecular formula was $C_{37}H_{45}NO_5$ ([M + H]$^+$ 584.33697) from the (+)-HR FABMS. Because of the presence of the unusual *N*-2-naphthyl-*N*-phenylamino substituent in compound **2**, which hitherto has not been described as a natural product, the crude extract of *Microsphaeropsis* sp. was analyzed by HPLC. *N*-Phenyl-2-naphthylamine, however, which is commercially available, could not be detected. It is assumed that compound **2** is a true natural product and not an artifact originating, for example, from chemical reaction of the respective betaenone precursor with extraneous *N*-phenyl-2-naphthylamine. The aliphatic region of the ^{1}H and ^{13}C NMR spectra of **2** resembles that of **1** (Tables 1 and 2), while the major difference is the occurrence of a complex set of signals in the aromatic region of **2** that belong to a naphthalene ring and a phenyl ring system as determined from COSY and HMBC spectra. The latter spectra afforded an unambiguous confirmation of the signal assignments, substituent positions, and total structure. In particular, long-range correlations of H-18 to C-1′ and C-1″ established the nature of the *N*-substituent, which also confirms its attachment at C-18. In compound **2**, the 18-methoxyl group of **1** has been replaced by an *N*-2-naphthyl-*N*-phenylamino group.

Compounds **5**−**7** were obtained as yellow-orange powders. The UV spectra with maxima at ca. 450, 290, and 224 nm suggested an anthraquinone as the basic structure. The isolated compounds are C-2-substituted congeners of 1,3,6,8-tetrahydroxyanthraquinones, and their NMR data are closely related to those of the known compound, noraverufanin (**8**) (Tables 4 and 5). The ^{13}C NMR spectra of compounds **5** and **6** showed resonances at ca. δ 189 (s) and δ 181 (s) indicative of a quinone and four aromatic hydroxyl-substituted carbons at ca. δ 160, δ 163, δ 164, and δ 165. In the sp^3 region, the presence of an O-substituted methine group was observed between 60 and 70 ppm with its corresponding methyl group at ca. 20 ppm.

The ^{1}H NMR data of compounds **5**−**7** were informative particularly when combined with the ^{13}C NMR data (**5** and **6**). The ^{1}H NMR spectrum of **5** showed only three proton

Table 3. HMBC Data of **1**

H no.	correlation with carbon atoms
H3	C1, C2, C4, C11, C12, C13, C15, C16, C19
H5	C4, C7, C16, C19
$H7_{eq}$	C5, C6, C8, C9, C20, C21
$H7_{ax}$	C6, C8, C20, C21
$H9_{ax}$	C8
$H9_{eq}$	C8,C10
H11	C1, C2,C3, C11[a]
H12	C14, C15
H13	C12, C14, C15
H14	C12, C13
H15	C12, C13, C14
H17	C16, C17[a], C18
H18	C16
H19	C2[b], C3, C4 C5, C16, C19[a]
H20	C6, C7, C8[b]
H21	C7, C8, C9, C10[b], C21[a]
H22	C18

[a] CH direct corrrelation. [b]These four-bond correlations were weak but unambiguously present.

Table 4. ^{13}C NMR Data of the Anthraquinone Congeners **5**, **6**, and **8**

C no.	**5**[a]	**6**[a]	**8**[b]
1	160.6 s	162.7 s	161.5
2	122.8 s	120.2 s	120.1
3	63.3 s	163.3 s	162.4
4	109.4 d	108.5 d	108.8
5	108.6 d	108.6 d	108.8
6	165.0 s	165.1 s	165.3
7	108.0 d	108.1 d	108.2
8	164.1 s	164.2 s	164.3
9	188.6 s	188.9 s	188.9
10	181.2 s	181.3 s	181.2
11	134.9 s	134.9 s	134.9
12	108.8 s	108.7 s	108.7
13	107.9 s	108.2 s	108.6
14	132.9 s	133.2 s	133.3
15	63.2 d	70.3 d	73.0
16	22.2 q	18.7 q	28.8
17		55.9 q	68.5

[a] Spectra were recorded in DMSO-d_6. [b] Taken from ref 24.

Table 5. ^1H NMR Data of the Anthraquinones **5**, **6**, and **7** in DMSO-d_6

H no.	**5**	**6**	**7**
1	12.70 s	12.80 s	12.80 s
4	7.07 s	7.20 s	7.21 s
5	7.09 d 2.3 Hz	7.09 d 2.3 Hz[a]	7.20 s
7	6.57 d 2.3 Hz	6.57 d 2.3 Hz[a]	
8	12.10 s	12.1 s	12.10 s
15	5.29 q 6.5 Hz	4.95 q 6.7 Hz	4.95 q 6.7 Hz
16	1.44 d 6.6 Hz	1.49 d 6.7 Hz	1.49 d 6.7 Hz
17		3.14 s	3.10 s

[a] Unambiguously assigned from HMBC spectrum.

resonances in the sp^2 region, two of which are doublets occurring at δ 6.57 and δ 7.09 with coupling constants of 2.3 Hz, indicative of their meta disposition in the ring system. Similarly, in the sp^3 region, the presence of a O-substituted methine group was observed as a quartet at 5.29 ppm, which couples with a methyl doublet at 1.44 ppm. The substitution at C-2 was confirmed by cross-peaks observed between the methine proton at C-15 with C-1 and C-3.

Compounds **6** and **7** differ from **5** in the presence of an aliphatic methoxyl substituent at the methine carbon, as evident by the presence of an additional methyl carbon signal at 56 ppm in the ^{13}C NMR spectrum of **6** and singlets at 3.14 and 3.10 ppm of the aliphatic methoxyl groups in the corresponding ^1H NMR spectra of **6** and **7**, respectively. Compound **6** is the methoxyl derivative of **5**, and this was confirmed from the HMBC spectrum. In the ^1H NMR spectrum of compound **7**, the meta-oriented doublets in the sp^2 region were replaced by a singlet at 7.2 ppm, which, by comparison with the ^1H shifts of the aromatic protons of **6**, is only compatible with the introduction of a hydroxyl group at C-7. Hence, **7** is a 1,3,6,7,8-pentahydroxyanthraquinone congener of **6**.

The elucidation of the absolute configuration of the 1,3,6,8-tetrahydroxyanthraquinones **5** and **6** was achieved by comparison of their experimental CD spectra with those from quantum chemical CD calculations, an efficient method that was established and improved by our group.[16−19] Semiempirical conformational analyses (PM3 parametrization) to obtain all relevant conformers for the calculation of the chiroptical properties were performed starting with the (*S*)-configured enantiomers.

All four hydroxy substituents of the anthraquinone ring were found to have two possible alignments each, differing by approximately 180° in their dihedral angles, whereas the substituent at C-2 showed five principal orientations, independent of the conformational behavior of the ring OH groups. Furthermore, the anthraquinone ring system has either a slightly convex or concave curvature, leading to an overall number of $(2^4) \times 5 \times 2 = 160$ minimum structures for each of the two 1,3,6,8-tetrahydroxyanthraquinones. For the calculation of the circular dichroism, however, only 28 conformers of (*S*)-**5** and four conformers of (*S*)-**6** with relative heats of formation no higher than 3 kcal/mol above the global minimum were considered. Due to the absence of one or several hydrogen bonds, the energies of the other minimum structures were too high so that their contributions to the overall CD spectra were negligible. For all energetically favored conformers, single CD spectra were calculated and weighted according to the Boltzmann statistic, i.e., according to the heats of formation of the corresponding conformers, and summed to give the overall CD spectra, which were subsequently "UV-corrected".[16] Compared with the experimental CD spectra of **5** and **6**, the calculated CD spectra of (*S*)-**5** and (*S*)-**6** were virtually opposite (see Figure 1a and b, left side), whereas the theoretical spectra of the (*R*)-configured enantiomers, as obtained by reflection of the calculated spectra of (*S*)-**5** and (*S*)-**6** about the zero line, showed a very good agreement with the experimental ones (see Figure 1a and b, right side). With these results, the absolute configuration of the two 1,3,6,8-tetrahydroxyanthraquinones **5** and **6** could be assigned unequivocally as (*R*). Because of the high similarity of the experimental CD spectra of **5** and **6** with those of **7** and the very close relationship of these three compounds, the 1,3,6,7,8-pentahydroxyanthraquinone **7** can likewise be attributed the (*R*)-configuration.

The unexpectedly low $\Delta\epsilon$ values measured for **5** and **6** may be indicative of a partial racemic character of these two compounds, possibly as a consequence of the configurational instability of the benzylic alcohol functionality. This was confirmed by a ruthenium-mediated oxidative degradation of **5** and **6** recently developed,[20] by conversion of the C_3 portion consisting of the C_2 side chain and the adjacent aromatic C atom, into lactic acid, which was found to be virtually racemic, so that the CD spectra of **5** and **6** must arise from the small excess of the respective (*R*)-enantiomers.

As yet, only a methyl-branched unsaturated fatty acid and its glyceride have been reported from the marine fungus *Microsphaeropsis olivacea*, which was obtained from a Florida sponge.[21] Terrestrial species of the genus *Mi-*

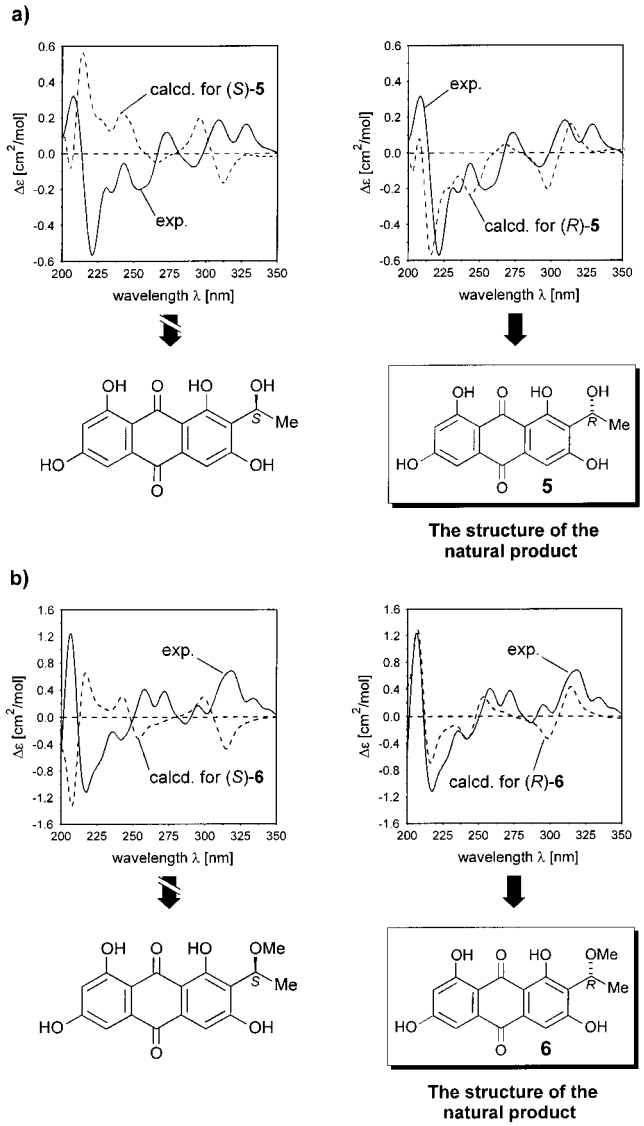


Figure 1. Attribution of the absolute configuration of (a) **5** and (b) **6**.

crosphaeropsis, however, have afforded a variety of bioactive secondary metabolites, which include lactam epoxides,[22] diketopiperazines,[23] and macrosphelides.[24,25]

The betaenones were first isolated as phytotoxic metabolites from *Phoma betae* Fr., the causal fungus of the leafspot disease of sugar beet.[11−13] Their structures are related to those of the stemphyloxins, phytotoxins produced by the plant pathogenic fungus *Stemphylium botryosum* Walr. F. sp. *lycopersici*.[26] These compounds have been shown to inhibit RNA and protein synthesis in vivo.[11−13,26] The isolation of 1,3,6,8-tetrahydroxyanthraquinones has been previously reported from the mycelium of *Aspergillus versicolor* (Vuillemin) Tiraboshi.[14]

Since many secondary metabolites have been described as inhibitors of protein kinases, we tested the new compounds for inhibition of protein kinases in vitro. These enzymes are of particular interest as targets for the development of novel anticancer drugs.[27] For a first assessment of the kinase inhibitory potential we selected an isozyme of the protein kinase C (PKC) family, the cyclin-dependent kinase 4 (CDK4) in complex with its activator cyclin D1, and the tyrosine kinase domain of the epidermal growth factor receptor (EGF-R). The relevance of all these kinases as pharmacological targets has been extensively described in the literature.[28−30]

Table 6. Inhibition of Protein Kinases by Anthraquinones and Betaenone Derivatives (Mean Values from Two Independent Experiments

	inhibition of protein kinases: IC_{50} (μM)		
compound	PKC-ϵ	CDK4/cyclin D1	EGF-R
1	36.0	11.5	10.5
2	≫100	≫100	≫100
5	18.5	43.5	37.5
6	27.0	22.5	27.5
7	54.0	37.5	41.0

Of the two betaenone derivatives found here, only **1**, which was tested as an isomeric mixture, inhibited all three kinases, whereas **2** showed no inhibition of PKC-, CDK4-, and EGF-R tyrosine kinase (Table 3). In contrast, each of the three anthraquinones inhibited all three kinases with similar potency. The range of the IC50 values was between 18.5 and 54.0 μM (Table 6).

The different inhibitory profiles found for the two betaenone derivatives suggest the enone side chain as a position for further optimization of such compounds toward higher potency and better selectivity. Similarly the three anthraquinones described in this paper may serve as lead structures for the development of more potent and selective protein kinase inhibitors.

Experimental Section

General Experimental Procedures. ^{1}H NMR and ^{13}C NMR spectra (chemical shifts in ppm) were recorded on Bruker ARX 400, DRX 500, or DMX 600 NMR spectrometers. Mass spectra (EI, APCI, CI) were measured on a Finnigan MAT 8430 and on an Intectra AMD 402 mass spectrometer. CD spectra were measured at room temperature in EtOH using the CD6 spectrometer from Jobin Yvon Instruments S.A. (France) for compounds **1**−**4** and **7** and the J-715 CD spectrometer from JASCO Deutschland (Gross-Umstadt, Germany) for compounds **5** and **6**. Optical rotations were recorded on a Perkin-Elmer-241 MC polarimeter. For HPLC analysis, samples were injected into a HPLC system coupled to a photodiode-array detector (Gynkotek, München, Germany). Routine detection was at 254, 280, and 340 nm. The separation column (125 × 4 mm, i.d.) was prefilled with Eurosphere C-18 (μm) (Knauer, Germany). Solvents were distilled before use, and spectral grade solvents were used for spectroscopic measurements. TLC plates with SiO_2 gel 60 F254 (Merck, Darmstadt, Germany) were used for monitoring the fractions. The compounds were detected by UV of 254 and 366 nm and also by spraying the TLC plates with anisaldehyde reagent. GC-EI-MSD analyses were performed on a Hewlett-Packard 5890 Series II GC with a Hewlett-Packard 5971A MSD. Transferline temperature was maintained at 280 °C, resulting in a source temperature of 170 °C and an ionizing energy of 70 eV. A β-DEX 325 (Supelco) 30 m × 0.25 mm (i.d.) × 0.25 μm column was used for chromatographic enantiomer separation of methoxypropionic acid. Analyses were performed in single ion monitoring-mode (SIM) with *m*/*z* 58. Helium was used as the carrier gas with a column head pressure of 80 kPa. Column temperature was finally programmed for 5 min at 100 °C, 5 °C min^{-1} to 150 °C for 5 min, 10 °C min^{-1} to 200 °C, and held for 5 min.

Microorganisms. The fungus *Microsphaeropsis* sp. was isolated from fresh samples of *Aplysina aerophoba*. Sponge samples were collected by scuba diving in the Mediterranean sea along the shores of Banyuls-sur-Mer in Southern France in the summer of 1996. Tissue samples were taken from inside the sponge body under sterile conditions and were reinoculated on malt agar slants that consisted of 15 g/L malt extract, 15 g/L agar, and 24.4 g/L artificial sea-salt mixture. The inoculated agar slants were then incubated at 27 °C. From the growing cultures a pure strain was isolated by repeated

inoculation on malt agar plates. This fungal strain was identified as *Microsphaeropsis* sp. by the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. The strain was deposited in the "Kulturensammlung Marine Pilze Bremerhaven (KMPB)" under the accession no. KMPB W-22. Its assignment was corroborated by further mycological investigations of the Marine Mycological Research Group of the Alfred-Wegener-Institute for Polar and Marine Research, Bremerhaven, Germany. However, due to the lack of authentic material for comparison, we could not identify the strain to species. It is thus possible that this fungus isolated from a marine sponge constitutes a new species. Until now 44 species of the genus *Microsphaeropsis* have been described worldwide. The genus *Microsphaeropsis* is classified within the formphylum Fungi imperfecti (Deuteromycota, Mitosporic Fungi) and belongs to the class Hyphomycetes in the traditional system, or to the Mitosporic Fungi, Code Group 4.A2.15, according to a modern system by Hawksworth.[31] This genus is characterized by the filamentous growth of its thallus and a holoblastic conidium ontogeny, with successively produced single phaeospores, which are schizolytically seceded and sometimes accumulate to pseudo-chains. Our strain KMPB W-22 produces increasingly pigmented intercalary chlamydospores on glucose-peptone-yeast extract-agar made up with tap water and a narrow aerial mycelium on Sabouraud-agar only.

Extraction and Isolation. Mass growth of the fungus for the isolation and identification of new metabolites was carried out in Erlenmeyer flasks at the Hans-Knöll-Institute, Jena. The fungus was grown in 1.5% malt-extract broth (ME) made up with distilled water supplemented with 2.44% of SERA artificial sea-salts mixture (S) at room temperature (no shaking). After 41 days the fungal biomass was separated from the culture broth, and both the mycelium and the broth were extracted with EtOAc. The extracts were combined and dispatched to the University of Würzburg for further chemical processing.

The total extract was evaporated under reduced pressure and taken to dryness (12.17 g). The residue was partitionated between EtOAc (400 mL × 4) and H_2O (400 mL). The organic fraction was again taken to dryness (10.71 g) and chromatographed over SiO_2 gel by vacuum liquid chromatography using a step-gradient method of elution employing CH_2Cl_2 and MeOH (100:0, 98:2, 95:5, 90:10, 80:20, 30:70) as solvent systems. Ten fractions were obtained; the lipophilic fractions 1 and 2 contained fatty acids, while fractions 3 and 4 contained both the betaenones and the anthraquinones. Fractions 3 and 4 were eluted at a solvent ratio of 95:5 and 90:10, respectively. Fractions 3 and 4 were pooled and chromatographed over a Sephadex LH 20 column using MeOH as eluting solvent from which 17 fractions were obtained. Fractions 6 and 7 contained the betaenone congeners **1**, **1a**, and **2**, while the anthraquinone derivatives **5**, **6**, and **7** were obtained from the yellow-colored fractions 11, 12, and 13. Purification of the betaenones was accomplished by rechromatography on RP18 Lobar columns. Compound **1** (17.8 mg) was obtained from fraction 7 with MeOH:H_2O:TFA (95:5:0.1) as eluent, while compound **2** (8.1 mg) was eluted from fraction 6 with MeOH:H_2O:TFA (90:10:0.1). The anthraquinone congeners **5** (12.7 mg), **6** (7.4 mg), and **7** (1.7 mg) were purified by semipreparative HPLC on a C18 column using the following gradient: 0 min, 80% MeOH; 20 min, 90% MeOH; 25−30 min, 100% MeOH.

Oxidative Degradation Procedure. The reaction was performed as previously described.[32] To a stirred mixture of 80 μL of CCl_4, 80 μL of MeCN, and 160 μL of 9.1 M Na−Pi buffer (pH 6, contains $NaH_2PO_4 \times H_2O$, NaOH) were added 2.7 mg (8.3 μmol) of **6** and the catalyst $RuCl_3 \cdot H_2O$ (0.1 mg, 0.4 μmol). To this solution 36 mg of $NaIO_4$ (170 μmol) was added at room temperature within 45 min in small portions. After stirring for another 1.5 h the resulting 2-methoxypropionic acid was extracted with 700 μL of H_2O and washed two times with 300 μL of $CHCl_3$. The aqueous layer was subsequently lyophilized and the residue dissolved in 1.5 mL of dry MeOH. The insoluble inorganic salts were separated by centrifugation and filtration over a microfilter. The resulting solution was dried and resolved in CH_2Cl_2 for the GC−MS analysis.

Conformational Analyses. All conformational analyses were performed on Silicon Graphics OCTANE R10000 workstations by means of the PM3[33] parametrization as implemented in the program package VAMP6.5,[34] starting from geometries preoptimized by the TRIPOS[35] force field.

CD Calculations. The wave functions for the calculation of the rotational strengths for the electric transitions from the ground state to excited states were obtained by CNDO/S-CI calculations[36] with a CI expansion including 576 singly occupied configurations and the ground state determinant. These calculations were carried out on LinuX PentiumII workstations by the use of the BDZDO/MCDSPD[37] program package. All single CD spectra thus obtained were added up following Boltzmann statistics using appropriate heats of formation to give the calculated overall CD spectrum. For a better visualization, the rotational strengths were transformed into $\Delta\epsilon$ values and superimposed with a Gaussian band shape function.

Production of Recombinant Protein Kinases. Protein kinases were cloned from RNA from appropriate cells by RT-PCR. Subsequently the isolated cDNAs were subcloned into the baculovirus transfer vectors pAcG or pAcGHLT from PharMingen (San Diego, CA) in order to express the protein kinases as GST-fusion proteins. Recombinant baculoviruses were produced with the BaculoGold system from the same manufacturer. Sf9 insect cells were infected with recombinant baculoviruses and harvested after 72 h. GST-fusion proteins were purified in a batch procedure using glutathione agarose beads as described (PharMingen manual). Recombinant proteins were characterized by silver staining of SDS gels and western blot analysis with kinase-specific antibodies and anti-GST antibodies.

PKC-ϵ and CDK4 were expressed as full-length protein kinases, whereas for EGF-R only the cDNA of the tyrosine kinase domain was used for protein expression. Cyclin D1 was coexpressed with CDK4. EGF-R tyrosine kinase, CDK4, and cyclin D1 were expressed as human sequences, whereas the cDNA for PKC-ϵ expression was cloned from mouse.

Protein Kinase Assays. All kinase assays were performed in 96-well flash plates from NEN (Boston, MA) in a 100 μL reaction volume. The PKC-ϵ assays contained 50 mM HEPES−NaOH, pH 7.5, 1 mM EDTA, 1.25 mM EDTA, 5 mM $MgCl_2$, 1.32 mM $CaCl_2$, 5 μg/mL phosphatidylserine, 1 μg/mL 1,2-diolein, 1 mM DTT, 0.1 μM [^{33}P]-(-ATP (5 × 105 cpm), 50 ng of protein kinase, and 0.5 μg of histone H1. EGF-R and CDK4 protein kinase assays contained 50 mM HEPES−NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM Na-orthovanadate, 1 mM DTT, 0.1 μM ATP, 100 ng of protein kinase, and 1 μg of poly(Glu,Tyr) 4:1 (EGF-R) or 1 μg of Rb protein (CDK4).

The reaction cocktails were incubated at 30 °C for 80 min. Reaction was stopped with 100 μL of 2% H_3PO_4, and plates were washed three times with H_2O. Incorporation of ^{33}P was determined with a microplate scintillation counter. Test compounds were dissolved at a concentration of 10 mM in 100% DMSO and further diluted in half-logarithmic steps. The final DMSO concentration in the assay was 1%.

10-Hydroxy-18-methoxylbetaenone (1): white powder; (R_f 0.71, CH_2Cl_2−MeOH; 95:5); $[\alpha]_D$ +16.6° (*c* 1.25 EtOH); UV (max) (MeOH) 265 nm; CD (EtOH) $\Delta\epsilon = -20$ (210 nm), −25.0 (220 nm); ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; ESI pos (4.0 kV) $[2M + Na]^+$ *m*/*z* 815.4 (basis peak), $[M + Na]^+$ *m*/*z* 419.2; molecular formula of $C_{22}H_{36}O_6$ determined from the high-resolution EI mass spectral data of the molecular ion $[M]^+$ (found, 396.2458; calculated, 396.2512).

10-Hydroxy-18-*N*-2-naphtyl-*N*-phenylaminobetaenone (2): white powder (R_f 0.72, CH_2Cl_2−MeOH; 95:5); $[\alpha]_D$ −44.4° (*c* 1.0, EtOH); UV (max) (MeOH) 221 and 350 nm; CD (EtOH) $\Delta\epsilon = -36$ (206.5 nm), −37.0 (220 nm); ^{1}H NMR, see Table 2; ^{13}C NMR, see Table 1; ESI pos (4.0 kV) $[M + Na]^+$ *m*/*z* 606 (basis peak), $[M + H]^+$ *m*/*z* 584; ESI neg (4.0 kV) $[M - H]^+$ *m*/*z* 582; molecular formula of $C_{37}H_{45}NO_5$ determined from the high-resolution ESI mass spectral data of the

molecular ion (found, $[M + H]^+$ 584.33697; calculated for $[M + H]^+$, 584.3375).

Compound 5: yellow-orange powder (R_f 0.75, CH_2Cl_2:MeOH; 95:5); $[\alpha]_D$ +151.5° (*c* 0.1, EtOH); UV (max) (MeOH) 224, 291, and 430 nm; CD (EtOH) $\Delta\epsilon = +0.41$ (208 nm), −0.73 (221 nm), −0.28 (235 nm), −0.25 (256 nm), +0.15 (272 nm), +0.24 (309 nm), +0.21 (329 nm); ^{1}H NMR, see Table 5; ^{13}C NMR, see Table 4; molecular ion peak $[M + H]^+$ at *m*/*z* 317 in CIMS, which is compatible with the molecular formula $C_{16}H_{12}O_7$.

Compound 6: yellow-orange powder (R_f 0.73, CH_2Cl_2−MeOH; 95:5); $[\alpha]_D$ −193.8° (*c* 0.1, EtOH); UV (max) (MeOH) 224, 292, and 453 nm; CD (EtOH) $\Delta\epsilon = +1.30$ (207 nm), −0.85 (217 nm), −0.21 (243 nm), +0.25 (258 nm), +0.24 (272 nm), 0.42 (318 nm); ^{1}H NMR, see Table 5; ^{13}C NMR, see Table 4; EIMS *m*/*z* $[M]^+$ 330 (12), 315 (8), 298 (100), 270 (16), ESI pos $[M + H]^+$ *m*/*z* 331, 214.3 (basis peak), ESI neg $[M - H]^-$ *m*/*z* 329.5, APCI pos $[M + H]^+$ *m*/*z* 331, 299 (basis peak); molecular formula of $C_{17}H_{14}O_7$ determined from the high-resolution (+)-ESI mass spectral data of the molecular ion (found, $[M - H]^-$ 329.0688; calculated, $[M - H]^-$ 329.0657).

Compound 7: yellow-orange powder (R_f 0.67, CH_2Cl_2−MeOH; 95:5); $[\alpha]_D$ −380.2° (*c* 0.1, EtOH); UV (max) (MeOH) 225, 295, 330, and 450 nm; CD (EtOH) $\Delta\epsilon = -19$ (212 nm), −15 (228 nm), +3 (246 nm); ^{1}H NMR, see Table 5; ^{13}C NMR, see Table 4; APCI pos MS/MS $[M + H]^+$ *m*/*z* 347.9 (36), 312.0 (80), 283.9 (100); molecular ion peak $[M + H]^+$ at *m*/*z* 347 in ESI-MS, which is compatible with the molecular formula $C_{17}H_{14}O_8$.

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References and Notes

(1) Gustafson, K.; Roman, M.; Fenical, W. *J. Am. Chem. Soc.* **1989**, *111*, 7519−7524.
(2) Tapiolas, D. M.; Roman, M.; Fenical, W.; Stout, T. J.; Clardy, D. J. *J. Am. Chem. Soc.* **1991**, *113*, 4682−4686.
(3) Trishman, J. A.; Jensen, P. R.; Fenical, W. *Tetrahedron Lett.* **1994**, *35*, 5571−5574.
(4) Trishman, J. A.; Tapiolas, D. M.; Jensen, P. R.; Dwight, R.; Fenical, W. *J. Am. Chem. Soc.* **1994**, *116*, 757−758.
(5) Pathirana, C.; Jensen, P. R.; Fenical, W. *Tetrahedron Lett.* **1992**, *33*, 7663−7666.
(6) Cheng, X. C.; Varoglu, M.; Abrell, L.; Crews, P.; Lobkovsky, E. *J. Org. Chem.* **1994**, *59*, 6344−6348.
(7) Varoglu, M.; Corbett, T. H.; Valeriote, F. A.; Crews, P. *J. Org. Chem.* **1997**, *62*, 7078−7079.
(8) Okutani, K. *Bull. Jpn. Soc. Sci.* **1977**, *43*, 995.
(9) Poch, G. K.; Gloer, J. B. *Tetrahedron Lett.* **1989**, *30*, 3483−3486.
(10) Poch, G. K.; Gloer, J. B. *J. Nat. Prod.* **1991**, *54*, 213−217.
(11) Ichihara, A.; Oikawa, H.; Hayashi, K.; Sakamura, S. *J. Am. Chem. Soc.* **1983**, *105*, 2907−2908.
(12) Ichihara, A.; Oikawa, H.; Hashimoto, M.; Sakamura, S.; Haraguchi, T.; Nagano, H. *Agric. Biol. Chem.* **1983**, *47*, 2965−2967.
(13) Oikawa, H.; Ichihara, A.; Sakamura, S. *Agric. Biol. Chem.* **1984**, *48*, 2603−2605.
(14) Berger, Y. *Phytochemistry* **1980**, *19*, 2779−2780.
(15) Castonguat, A.; Berger, Y. *Tetrahedron* **1979**, *35*, 1557−1563.
(16) Bringmann, G.; Busemann, S. In *Natural Product Analysis*; Schreier, P., Herderich, M.;, Humpf, H. U., Schwab, W., Eds.; Vieweg: Braunschweig, 1998; pp 195−212.
(17) Bringmann, G.; Günther, C.; Busemann, S.; Schäffer, M.; Olowokudejo, J. D.; Alo, B. *Phytochemistry* **1998**, *47*, 37−43.
(18) Linker, T.; Rebien, F.; Tóth, G.; Kraus, J.; Bringmann, G. *Chem. Eur. J.* **1998**, *4*, 1944−1951.
(19) Bringmann, G.; Kraus, J.; Menche, D.; Messer, K. *Tetrahedron* **1999**, *55*, 7563−7572.
(20) Bringmann, G.; Münchbach, M.; Michel, M. Unpublished results.
(21) Yu, C.; Curtis, J. M.; Wright, J. L. C.; Ayer, S. W. *Can. J. Chem.* **1996**, *74*, 730−735.
(22) Tony Lam, Y. K.; Hensens, O. D.; Ransom, R.; Giacobbe, R. A.; Polishook, J.; Zink, D. *Tetrahedron* **1996**, *52*, 1481−1486.
(23) Funabashi, Y.; Horiguchi, T.; Iinuma, S.; Tanida, S.; Harada, S. *J. Antibiot.* **1994**, *47*, 1202−1218.
(24) Takamatsu, S.; Kim, Y.; Hayasi, M.; Hiraoka, H.; Natori, M.; Komiyama, K.; Omura, S. *J. Antibiot.* **1996**, *49*, 95−99.
(25) Hayasi, M.; Kim, Y.; Hiraoka, H.; Natori, M.; Takamatsu, S.; Kawakubo, T.; Masuma, R.; Komiyama, K.; Omura, S. *J. Antibiot.* **1995**, *48*, 1435−1439.
(26) Barash, I.; Kashman, Y. K.; Clardy, J.; Strobes, G. A. *Science* **1983**, *25*, 1065−1066.
(27) Patrick, D. R.; Heimbrook, D. C. *Drug Discovery Today* **1996**, *1*, 325−330.
(28) Hu, H. *Drug Discovery Today* **1996**, *1*, 438−447.
(29) Rao, R. N. *Curr. Opinion Oncol.* **1996**, *8*, 516−524.
(30) Levitzki, A.; Gazit, A. *Science* **1995**, *267*, 1782−1788.
(31) Hawksworth, D. L.; Kirk, P. M.; Sutton, B. C.; Pegler, D. N. *Ainsworth & Bisby's Dictionary of the Fungi*, 8th ed.; CAB International: Wallingford, 1995; 616 pp.
(32) Bringmann, G.; God, R.; Schäffer; M. *Phytochemistry* **1996**, *43*, 1393−1403.
(33) Steward, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209−264.
(34) Rauhut, G.; Chandrasekhar, J.; Alex, A.; Beck, B.; Sauer, W.; Clark, T. *VAMP 6.5*; Oxford Molecular Ltd.: The Medewar Centre, Oxford Science Park, Sandford-on-Thames, Oxford, OX4 4GA, England.
(35) *SYBYL*; Tripos Associates: 1699 St. Hanley Rd., Suite 303, St. Louis, MO, 63144.
(36) Del Bene, J.; Jaffé, J. J. *Chem. Phys.* **1968**, *48*, 1807−1813.
(37) Downing, J. W. Program Packet *BDZDO/MCDSPD*; Department of Chemistry and Biochemistry, University of Colorado: Boulder, CO; modified by Fleischhauer, J.; Schleker, W.; Kramer; B.; ported to LinuX by Gulden, K.-P.

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