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Structural and Conformational Analysis of Proanthocyanidins from *Parapiptadenia rigida* and Their Wound-Healing Properties

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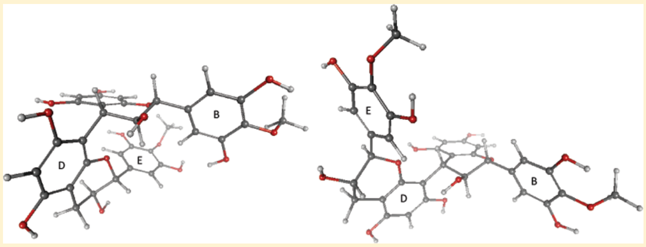
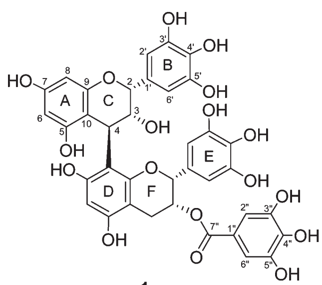
S *Supporting Information*

ABSTRACT: Structure elucidation and conformation analysis of four proanthocyanidins isolated from the bark of *Parapiptadenia rigida* were performed by two-dimensional NMR spectroscopy, HRESIMS, CD, and molecular mechanics (MM+) force field calculations. The known prodelphinidin, epigallocatechin-(4β→8)-epigallocatechin-3-*O*-gallate (**1**) was accompanied by the new epigallocatechin-(4β→8)-4′-*O*-methylgallocatechin (**2**), epicatechin-(4β→8)-4′-*O*-methylgallocatechin (**3**), and (4α→8)-bis-4′-*O*-methylgallocatechin (**4**). Compound **4** was previously published but the earlier structure must presumably be revised to 4′-*O*-methylgallocatechin-(4α→8)-4′-*O*-methylepigallocatechin. Conformational studies showed the compact rotamer with B and E rings in quasi-equatorial orientations as the preferred conformation for compounds **1**−**3**, whereas **4** consists of two stable rotamers, each with a quasi-equatorial orientation of ring B and E, respectively. The isolated compounds were studied for their wound-healing effects in a scratch assay and showed promising results.



Recently we reported the structure elucidation of the monomeric catechin derivatives from the bark of *Parapiptadenia rigida* (Benth.) Brenan (Fabaceae).[1] Preparations from its bark are used in Brazilian traditional medicine because of its wound-healing, anti-inflammatory, astringent, expectorant, antidiarrheic, antihemorrhagic, and antimicrobial properties.[2−4] Continuation of our investigation on the ethanolic extract led to the isolation of four dimeric proanthocyanidins among which the two dimeric prodelphinidins (**2**, **4**) and the heterogeneous procyanidin (**3**) are described here for the first time. Moreover, extensive conformational analysis of the isolated molecules was performed to gain details of their conformational behavior. Proanthocyanidins are widely distributed in the plant kingdom and are considered as one of the most abundant groups of natural phenolics.[5,6] These molecules possess a high antioxidative potential and are thought to have beneficial effects especially in diseases related with oxidative stress and free radicals.[7,8] As they are also described to facilitate wound-healing,[9] all compounds except **1** were studied in cell-based assays for their wound-healing properties in a scratch assay and in the NF-κB EMSA.



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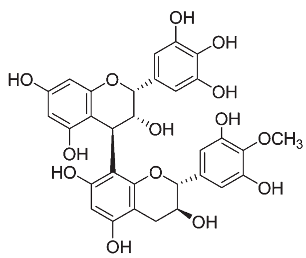

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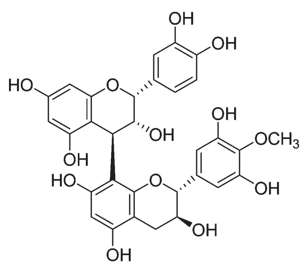

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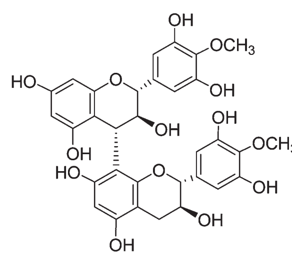

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■ RESULTS AND DISCUSSION

Fractionation of the ethanolic bark extract from *P. rigida* afforded **1**. On the basis of one-dimensional (1D) and 2D NMR data (^{1}H, ^{13}C, COSY, HSQC, HMBC), MS (ESI) as well as optical rotation data, it was identified as epigallocatechin-(4β→8)-epigallocatechin-3-*O*-gallate, a prodelphinidin that was first isolated

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Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Data for 2 and 3 in Methanol-d_4, 248 K (δ in ppm, J in Hz)

	2			3		
position	δ_C, type	δ_H (J in Hz)	HMBC[a]	δ_C, type	δ_H (J in Hz)	HMBC[a]
2C	77.0, CH	4.99, brs	1′B, 2′B, 6′B	75.7, CH	5.09, s	1′B, 2′B, 6′B
3C	73.0, CH	3.89, brs	10A	71.6, CH	3.91, brs	2C, 10A
4C	36.7, CH	4.64, brs	3C, 2C, 7D, 8D, 9D, 10A	35.4, CH	4.66, brs	3C, 2C, 7D, 8D, 9D, 5A, 9A, 10A
5A	158.1, qC			156.7, qC		
6A	95.9, CH	5.93, d (2.2)	5A,7A, 8A, 10A	94.5, CH	5.96, brs	5A,7A, 8A, 10A
7A	158.6, qC			157.3, qC		
8A	95.4, CH	6.96, d (2.2)	6A, 7A, 9A, 10A	94.0, CH	5.97, brs	6A, 7A, 9A, 10A
9A	157.8, qC			156.4, qC		
10A	101.8, qC			100.5, qC		
1′B	131.8, qC			131.3, qC		
2′B	106.4, CH	6.38, s	2C, 1′B, 3′B, 4′B, 6′B	113.6, CH	6.89, d (1.2)	2C, 3′B, 4′B, 6′B
3′B	146.5, qC			144.4, qC		
4′B	133.2, qC			144.1, qC		
5′B	146.5, qC			114.3, CH	6.73, d (8.2)	
6′B	106.4, CH	6.38, s	2C, 1′B, 2′B, 4′B, 5′B	117.8, CH	6.69, dd (1.2, 8.2)	2C, 1′B, 2′B, 4′B
2F	82.1, CH	4.80, d (6.1)	3F, 9D, 1′E, 2′E, 6′E	81.7, CH	4.83, d (6.1)	3F, 9D, 1′E, 2′E, 6′E
3F	68.4, CH	4.07, ddd (4.7, 6.1, 6.6)		66.9, CH	4.1, ddd (4.9, 6.1, 6.6)	10D
4F	27.4, CH_2	α 2.73, dd (4.7, 16.2)	9D	25.9, CH_2	α 2.74, dd (4.9, 16.3)	9D, 10D
		β 2.59, dd (6.6, 16.2)	3F, 9D, 10D		β 2.61, dd (6.6, 16.3)	3F, 5D, 10D
5D	155.8, qC			154.4, qC		
6D	96.8, CH	5.84, s	5D, 7D, 8D, 10D	95.4, CH	5.87, s	5D, 7D, 8D, 10D
7D	156.5, qC			155.1, qC		
8D	107.6, qC			106.2, qC		
9D	153.8, qC			152.4, qC		
10D	100.3, qC			98.9, qC		
1′E	137.1, qC			132.7, qC		
2′E	106.8, CH	6.51, s	2F, 1′E, 3′E, 4′E, 6′E	105.5, CH	6.53, s	2F, 1′E, 3′E, 4′E, 6′E
3′E	151.6, qC			150.2, qC		
4′E	136.0, qC			134.6, qC		
5′E	151.6, qC			150.2, qC		
6′E	106.8, CH	6.51, s	2F, 1′E, 2′E, 4′E, 5′E	105.5, CH	6.53, s	2F, 1′E, 2′E, 4′E, 5′E
O-Me (4′E)	60.7, CH_3	3.75, s	4′E	59.3, CH_3	3.77, s	4′E

[a] HMBC correlations are from proton(s) stated to the indicated carbon.

from the bark of *Myrica rubra*[10] and since then also from other plants.[11−17] However, as yet no unambiguous full assignment of the ^{1}H and ^{13}C NMR spectra has appeared and is reported here for the first time.

Fractionation of the ethanolic extract yielded three new proanthocyanidins that differ in their *O*-methylation patterns from known ones. NMR analyses have been performed on underivatized compounds. In most cases, full NMR data is unavailable for the parent nonmethylated precursors or only exists for acetylated derivatives.

Compound **2** was obtained as brownish-colored solid. Its molecular formula was deduced as $C_{31}H_{29}O_{14}$ from the HRESIMS ion at m/z 625.15503 $[M + H]^+$. At ambient temperature, broadened proton signals and only a few carbon resonances, incompatible with the MS data, were observed in the NMR spectra due to atropisomerism that results from steric interactions in the vicinity of the interflavanyl bond in proanthocyanidins.[18] Hence, NMR spectra (Table 1) were recorded at low temperature (248 K) where conformational exchange is almost completely frozen resulting in two sets of sharp resonances. Under these conditions, the typical profile of flavan-3-ols was apparent in the NMR spectra.[19]

The ^{1}H NMR spectrum of **2** showed one pair of methylene protons (H-4F) at δ_H 2.59 and 2.73 connected to a carbon signal at δ_C 27.4 (C-4F) as observed in the HSQC spectrum. A methine carbon at δc 36.7 was identified as C-4 of ring C. Its chemical shift and that of H-2C at δ_H 4.99 indicated that the interflavanoid linkage occurred at C-4C.[20] The upper unit was identified as epigallocatechin, as H-2C (δ_H 4.99) and H-3C (3.89) appeared as broad singlets characteristic of *cis*-orientation of these protons and an aromatic two-proton singlet at δ_H 6.38 with long-range correlation to C-2C (δ_C 77.02), indicating a trisubstituted B ring. Disubstitution of the A ring was confirmed by two aromatic doublets for H-6A (δ_H 5.93) and H-8A (5.96). The lower unit was identified as 4′-*O*-methylgallocatechin from the chemical shifts of H-2F (δ_H 4.8, d) and H-3F (4.07, ddd) with $J_{2,3}$ = 6.1 Hz characteristic of their *trans*-orientation and a two-proton singlet (δ_H 6.51) from the trisubstituted E-ring. The methoxy group (δ_C 60.7) was connected at C-4′E (136.0) from the long-range correlation between C-4′E and the three-proton singlet at

Table 2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Data for Rotamers 1 and 2 of 4 in Methanol-d_4, Room Temp (δ in ppm, J in Hz)

	rotamer 1			rotamer 2		
position	δ_C, type	δ_H (J in Hz)	HMBC[a]	δ_C, type	δ_H (J in Hz)	HMBC[a]
2C	82.6, CH	4.24, d (9.7)	1′B, 2′B, 6′B, 3C, 4C	82.6, CH	4.36, d (8.1)	1′B, 2′B, 6′B, 3C, 4C
3C	72.4, CH	4.33, m	2C, 10A, 8D	72.3, CH	4.54, m	10A, 2C, 4C, 8D
4C	37.2, CH	4.43, d (7.8)	3C, 10A, 8D,9D, 7D	37.0, CH	4.54, d (8.1)	3C, 7D, 8D, 9D, 5A, 9A, 10A
5A	156.5, qC			156.5, qC		
6A	96.2, CH	5.84, d (2.4)	7A, 8A, 10A	95.5, CH	5.79, d (2.4)	5A, 8A, 10A
7A	157, qC			157.0, qC		
8A	94.7, CH	5.88, d (2.4)	9A, 10A	96.3, CH	5.90, d (2.4)	6A, 7A, 9A, 10A
9A	155.4, qC			155.4, qC		
10A	105.7, qC			106.8, qC		
1′B	135.5, qC			135.3, qC		
2′B	107.1, CH	6.39, s	2C, 1′B, 3′B, 4′B, 6′B	107.2, CH	6.58, s	2C, 1′B, 3′B, 4′B, 6′B
3′B	149.7, qC			150.0, qC		
4′B[b]	134.9, qC			134.9, qC		
5′B	149.7, qC			150.0, qC		
6′B	107.1, CH	6.39, s	2C, 1′B, 2′B, 4′B, 5′B	107.2, CH	6.58, s	2C, 1′B, 2′B, 4′B, 5′B
O-Me (4′B)	59.6, CH_3	3.83, s		59.6, CH_3	3.83, s	
2F	80.8, CH	4.60, d (6.4)	3F, 4F, 9D, 1′E, 2′E, 6′E,	81.1, CH	4.75, d (6.7)	3F, 9D, 1′E, 2′E, 6′E
3F	66.9, CH	3.89, ddd (6.4, 5.1, 7.3)		67.0, CH	4.10, ddd (6.7, 5.1, 7.6)	
4F	26.6, CH_2	α 2.73, dd (5.1, 16.3)	2F, 3F, 5D, 9D, 10D	26.4, CH_2	α 2.81, dd (5.1, 16.2)	2F, 3F, 5D, 9D, 10D
		β 2.53, dd (7.3, 16.3)	3F, 5D, 9D, 10D		β 2.61, dd (7.6, 16.2)	2F, 3F, 5D, 10D
5D	154.3, qC			154.1, qC		
6D	94.4, CH	6.10, s	5D, 7D, 8D, 10D	96.1, CH	5.96, s	5D, 7D, 8D, 10D
7D	154.4, qC			154.2, qC		
8D	105.9, qC			106.9, qC		
9D	153.1, qC			153.3, qC		
10D	100.5, qC			98.9, qC		
1′E	134.8, qC			135.1, qC		
2′E	106.3, CH	6.10, s	2F, 1′E, 3′E, 4′E, 6′E	106.0, CH	6.55, s	2F, 1′E, 3′E, 4′E, 6′E
3′E	149.5, qC			150.1, qC		
4′E[b]	135.0, qC			135.2, qC		
5′E	149.5, qC			150.1, qC		
6′E	106.3, CH	6.10, s	2F, 1′E, 3′E, 4′E, 6′E	106.0, CH	6.55, s	2F, 1′E, 2′E, 4′E, 5′E
O-Me (4′E)	59.6, CH_3	3.82, s		59.6, CH_3	3.81, s	

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] Assignments are interchangeable for rotamers 1 and 2.

δ_H 3.75. The C-4→C-8 interflavan linkage was confirmed by the HMBC correlations between H-4C (δ_H 4.64) and C-7D (156.5), C-8D (107.6), and C-9D (153.8). C-9D was unequivocally assigned by its long-range correlations with H-2F (δ_H 4.80), H-4βF (2.59), and H-4αF (2.73). Therefore, the singlet at δ_H 5.84 represented H-6D. Consequently, C-5D (δ_C 155.8) and C-7D (156.5) were unambiguously assigned from their long-range correlations with H-6D. The relative positions of C-7D, C-5D, and C-9D, assigned in order of increasing field, agreed with NMR data reported for catechins and their derivatives.[21−23] The observed long-range correlations of H-4C with C-9A and C-5A confirmed their positions and corroborated the assignment of C-7A (δ_C 158.6), C-5A (158.1), and C-9A (157.8). The orientation of the C-4 flavanyl unit was assigned to be β (quasi-axial orientation) according to the chemical shift for C-2C at δ_C 77.0.[20] In the case of an α-orientation, a much larger downfield C-2C shift would have been expected as described for procyanidins B-3 and B-4 (δ_C 82 to 83). The resonances for H-3C and H-4C occurred as broad singlets and did not permit determination of the $J_{3,4}$ value. In the case of a coupling constant of about 8 Hz, an α-orientation would have been expected.[20,24] The positive Cotton effect in the 210−240 nm region of the CD spectrum confirmed a 4β-flavanyl substituent with a 4R configuration.[25−27] Thus, **2** was identified as the new prodelphinidin epigallocatechin-(4β→8)-4′-O-methylgallocatechin. The unmethylated prodelphinidin B-1 has been identified in some plant species[11,28−31] but with incomplete ^{1}H NMR and ^{13}C NMR data.

Compound **3** was isolated as a brownish solid. The ESIMS exhibited quasimolecular ions at m/z 609 $[M + H]^+$ in the positive mode and at m/z 607 $[M - H]^-$ in the negative mode consistent with the molecular formula of $C_{31}H_{28}O_{13}$. This was confirmed by the HRESIMS, which showed an $[M + H]^+$ ion at m/z 609.1603. NMR spectra showed atropisomerism at room

Table 3. Conformer Distribution of 1−4, Their Measured $J_{2,3}$ and $J_{3,4}$ Compared with Their Estimated Coupling Constants and Predicted Orientations of B and E Rings Determined for Each Lowest Energy-Minimized Conformation (LEC), as Well as Their Interflavan Bond Angles[a]

			B ring position	E ring position	
$J_{2,3}$; $J_{3,4}$ measured	LEC (energy: kcal mol^{-1})	*n*	ϕ H-2,3 (J)[b] ϕ H-3,4 (J)[b]	ϕ H-2,3 (J)[b] ϕ H-3,4α/β (J)[b]	interflavan angle ϕ = C(3)−C(4)-D(8)-D(9)
			Compound 1		
			eq	**eq**	
upper:	**1.1**	**97**	**-66.7 (1.4)**	**-69.8 (1.1)**	**+94.6° (compact)**
$J_{2,3}$ = br s	**(4.29)**		**-79.9 (0.6)**	**49.2 (3.3)/−68.4 (1.2)**	
$J_{3,4}$ = br s					
			ax	eq	
lower:	1.2	3	49.4 (3.3)	−67.5 (1.3)	+76.4° (compact)
$J_{2,3}$ = br s	(8.03)		−168.9 (9.1)	46.8 (3.6)/−70.7 (1.1)	
$J_{3,4\alpha}$ = 4.4					
$J_{3,4\beta}$ = 2.5					
			Compound 2		
			eq	**eq**	
	2.1	**54**	**-67.3 (1.3)**	**175.1 (9.3)**	**+95.8° (compact)**
upper:	**(3.1)**		**-78.6 (0.6)**	**44.7 (3.9)/162.8 (8.7)**	
$J_{2,3}$ = br s			ax	eq	
$J_{3,4}$ = br s	2.2	39	56.4 (2.5)	174.6 (9.3)	+81.3° (compact)
lower:	(4.9)		−164.2 (8.8)	42.3 (4.2)/160.3 (8.4)	
$J_{2,3}$ = 6.1			eq	ax	
$J_{3,4\alpha}$ = 4.7	2.3	7	−67.2 (1.3)	−68.6 (1.2)	+95.2° (compact)
$J_{3,4\beta}$ = 6.6	(5.4)		−79.9 (0.6)	−47.1 (3.6)/70.0 (1.1)	
			Compound 3		
			eq	**eq**	
	3.1	**65**	**-67.3 (1.3)**	**175.2 (9.3)**	**+95.8° (compact)**
upper:	**(4.5)**		**-78.6 (0.6)**	**44.8 (3.9)/162.8 (8.7)**	
$J_{2,3}$ = s			ax	eq	
$J_{3,4}$ = br s	3.2	32	56.8 (2.4)	174.7 (9.3)	+81.5° (compact)
lower:	(6.4)		−164.5 (8.8)	42.2 (4.2)/160.2 (8.4)	
$J_{2,3}$ = 6.1			eq	ax	
$J_{3,4\alpha}$ = 4.9	3.3	3	−68.7 (1.2)	−68.6 (1.2)	+95.4° (compact)
$J_{3,4\beta}$ = 6.6	(7.0)		−79.5 (0.6)	−47.1 (3.6)/69.8 (1.1)	
			Compound 4		
			ax	eq	
	4.1	10	−62.5 (1.8)	175.8 (9.3)	−106.8°(compact)
	(8.2)		77.6 (0.65)	46.7 (3.7)/164.8 (8.8)	
			eq	**eq**	
rotamer 1	**4.2**	**24**	**-177.2 (9.4)**	**175.6 (9.3)**	**-73.8° (compact)**
upper:	**(8.3)**		**159.6 (8.1)**	**43.7 (4.0)/161.8 (8.6)**	
$J_{2,3}$ = 9.7			eq	ax	
$J_{3,4}$ = 7.8	4.3	19	179.8 (9.4)	−68.8 (1.2)	−73.5° (compact)
lower:	(10.2)		160.6 (8.5)	−46.6 (3.7)/70.4 (1.1)	
$J_{2,3}$ = 6.4			ax	eq	
$J_{3,4\alpha}$ = 5.1	4.4	9	−60.0 (2.0)	176.6 (9.4)	+68.9° (extended)
$J_{3,4\beta}$ = 7.3	(11.4)		69.4 (1.2)	48.3 (3.5)/166.7 (8.9)	
			ax	ax	
rotamer 2	4.5	2	−61.9 (1.9)	−72.1 (1.0)	−108.4° (compact)
upper:	(11.5)		65.7 (1.5)	−40.3 (4.5)/76.3 (0.7)	
$J_{2,3}$ = 8.1					

Table 3. Continued

$J_{2,3}$; $J_{3,4}$ measured	LEC (energy: kcal mol^{-1})	n	B ring position: ϕ H-2,3 $(J)^b$ ϕ H-3,4 $(J)^b$	E ring position: ϕ H-2,3 $(J)^b$ ϕ H-3,4α/β $(J)^b$	interflavan angle ϕ = C(3)−C(4)-D(8)-D(9)
			eq	**eq**	
$J_{3,4}$ = 8.1	**4.6**	**14**	**178.3 (9.4)**	**176.4 (9.4)**	**+101.0° (extended)**
lower:	**(11.7)**		**165.5 (8.9)**	**47.3 (3.6)/165.6 (8.9)**	
$J_{2,3}$ = 6.7			ax	ax	
$J_{3,4\alpha}$ = 5.1	4.7	10	−62.1 (1.8)	−67 (1.4)	+73.8° (extended)
$J_{3,4\beta}$ = 7.6	(11.7)		71 (1.0)	−38.5 (4.7)/78.1 (0.6)	
			eq	ax	
	4.8	12	177.3 (9.4)	−62.1 (1.8)	+110.3° (extended)
	(12.4)		169.9 (9.1)	−47.1 (3.6)/70.4 (1.1)	

[a] Conformers that agree best with the NMR data are given in bold. ϕ H-2,3: H(2)−C(2)−C(3)−H(3); ϕ H-3,4: H(3)−C(4)−C(4)−H(4). [b] J estimated from ϕ of H-2,3 and H-3,4.

temperature as described for **2**. Therefore, measurement was again performed at 248 K. ^{1}H and ^{13}C NMR were similar to **2** except for resonances of an aromatic ABX-system at δ_H 6.69 (H-6′B, dd, J = 1.2, 8.2 Hz), 6.73 (H-5′B, d, J = 8.2 Hz), and 6.89 (H-2′B, d, J = 1.2 Hz) (Table 1) indicating epicatechin as the upper unit. The interflavanoid linkage was confirmed to be 4-C→8-D due to the HMBC correlations between H-4C (δ_H 4.66) and C-7D (155.1), C-8D (106.2), and C-9D (152.4). The resonance for C-9D was assigned according to its HMBC correlation with H-2F (δ_H 4.83). The 4β-linkage (4*R*) of the dimer was indicated by the positive Cotton effect observed in the 210−240 nm region of the CD spectrum of **3**.[25−27] Hence, **3** is the new heterogeneous procyanidin epicatechin-(4β→8)-4′-*O*-methylgallocatechin. The unmethylated compound has been reported from *Alhagi sparsifolia*,[32] *Phyllanthus emblica*,[33] and *Apocynum venetum*,[34,35] although no ^{13}C NMR data was given.

Compound **4**, obtained as a pale brown colored solid, showed quasimolecular ions at m/z 637 $[M - H]^-$ in the negative mode and a sodium adduct ion at m/z 662 $[M + Na]^+$ in the ESIMS. Consequently, an ion at m/z 639.1702 $[M + H]^+$ was observed in the HRESIMS from which a molecular formula of $C_{32}H_{30}O_{14}$ was concluded. Surprisingly, ^{1}H and ^{13}C NMR spectra recorded at room temperature or 248 K exhibited resonances for four flavan-3-ol moieties with similar intensities (see Table 2). This phenomenon can be explained by the occurrence of two atropisomers in a 1:1 ratio. Consequently, four resonances for each C-2, C-3, and C-4 were detected. The C-2 resonances in the range δ_C 80.8−82.6, together with large $J_{2,3}$ values (6.4 and 6.7, 9.7, and 8.1 Hz), were in agreement with a 2,3-*trans* relative configuration. In the HSQC spectrum, a pair of upfield methylene resonances (δ_C 26.4 and 26.6) and a downfield pair of methine resonances (δ_C 37.0 and 37.2) allowed identification of the C-4F methylene functionality. The two downfield methine resonances were consistent with an interflavanyl linkage between C-4 and either C-6 or C-8. Four aromatic two-proton singlets at δ_H 6.39, 6.10, 6.58, and 6.55 were identified as those of rings B and E of atropisomers 1 and 2, respectively, and indicated their trisubstitution. This was further confirmed by the HMBC correlations between H-2′ and H-6′ of rings B and E and C-2C and C-2F of the two atropisomers. In each pyrogallol ring, C-4′ carried an *O*-methyl group according to the long-range correlation with the three-proton singlet at δ_H 3.83 (6H), 3.82 (3H), and 3.81 (3H). *O*-methylation at the same position correlated well with the occurrence of a resonance for the methoxy group at δ_C 59.6. Additionally, two disubstituted A rings with resonances for H-6 (δ_H 5.84, d and 5.79, d) and H-8 (5.88, d and 5.90, d) and two trisubstituted D rings were observed. Trisubstitution was deduced from the two one-proton aromatic singlets (H-6: δ_H 6.10; 5.96) and two substituted aromatic carbons (C-8D: δ_C 105.9; 106.9). The interflavanoid linkage of rotamer 1 was determined to be C-4→C-8 from the long-range correlation between H-4C (δ_H 4.43) and C-7D (154.4), C-8D (105.9) and C-9D (153.1). For rotamer 2, HMBC correlations were observed between H-4C (δ_H 4.54) and C-7D (154.2), C-8D (107.0) and C-9D (153.3). In both cases, C-9 was unambiguously assigned from long-range correlations with H-2F (δ_H 4.60) in rotamer 1 and 4.75 in rotamer 2. The configuration of the interflavanoid linkages was evaluated using the same strategy as described for **2**. However, in this case both signals for C-2C were shifted downfield (δ_C 82.6) compared to the analogous C-2C in **2** (δ_C 77). Moreover, $J_{3,4}$ (7.8 and 8.1 Hz for rotamers 1 and 2, respectively) are characteristic of a substituent at C-4C with a quasi-equatorial (α) orientation.[20] The 4α-flavanyl substitution, equating with 4*S* absolute configuration was confirmed by the negative Cotton effect in the 210 − 240 nm region of the CD spectrum.[25−27] Therefore, **4** consists of two 4′-*O*-methylgallocatechin units with 4α→8 interflavanoid linkages. Evidence for the occurrence of two stable rotamers were further provided by measurement of a 2D ROESY spectrum, which showed several dipolar interactions between protons located on different rotamers at room temperature. These dipolar interactions disappeared when the spectrum was recorded at 248 K. However, all proton resonances from both rotamers remained visible with unchanged chemical shifts. All MS and NMR data agreed well with the occurrence of **4** as a mixture of two stable rotamers of 4′-*O*-methylgallocatechin-(4α→8)-4′-*O*-methylgallocatechin.

A proanthocyandin isolated from the bark of *Stryphnodendron adstringens* has been assigned the same structure.[36] However, our NMR data and those reported for gallocatechin-(4α→8)-gallocatechin (δ_C 83.1 and 83.9 for C-2B and C-2F)[37] are not in agreement with those published by de Mello et al.[36] A broadened overlapping resonance at δ_H 5.02 representing H-2(F) and H-3(F) and a shielded C-2 (δ_C 77.8) were reported for the lower catechin unit. These conflicting NMR data can either be

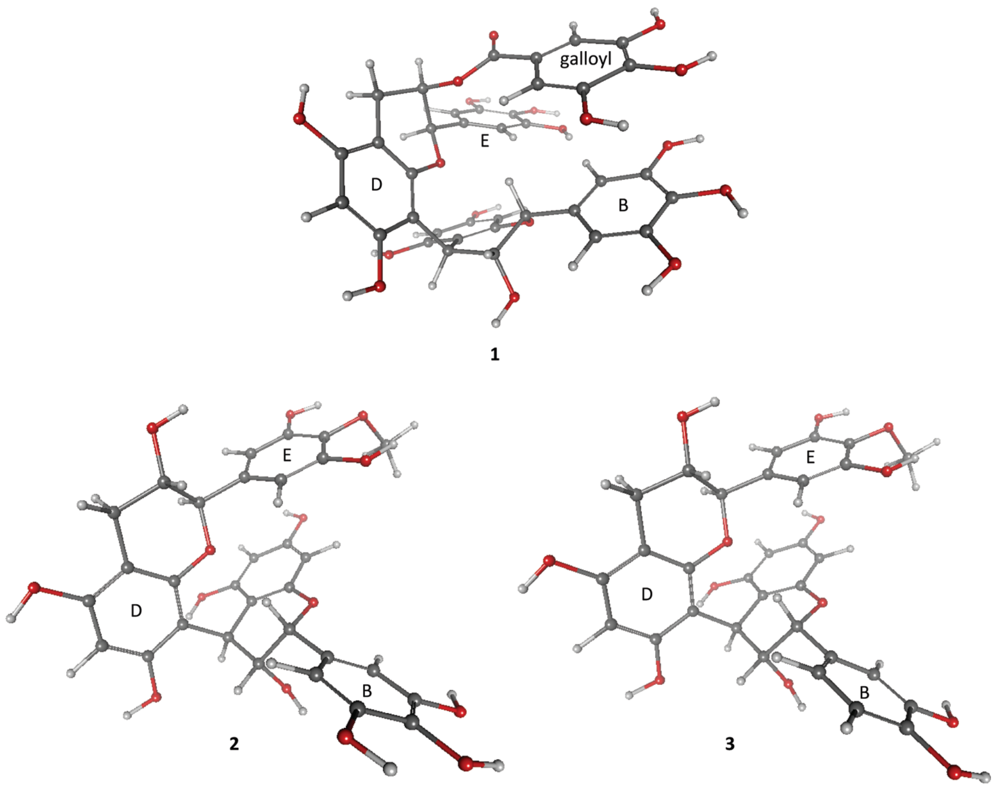

Figure 1. Energy-minimized structures proposed for **1**, **2**, and **3** with a compact conformation [ϕ C(3)−C(4)−D(8)−D(9) = positive] and rings B and E in quasi-equatorial orientation.

explained that the dimer from *S. adstringens* differs in conformation and is an eq/ax dimer[38] or, more likely, that the structure should be revised to 4′-*O*-methylgallocatechin-(4α→8)-4′-*O*-methylepigallocatechin. Although the NMR data have been obtained from the peracetylated derivative the reported $J_{2,3}$ values are more suitable for an epicatechin derivative, as catechins have broad splitting patterns for $J_{2,3}$. Moreover, the authors concluded the structure of lower terminal constituent from TLC analysis after acidic hydrolysis. However, the subfraction used for hydrolysis also contained epigallocatechin. Therefore, no conclusion can be drawn from this analysis. Fletcher et al.[20] demonstrated for procyanidins B-3 and B-4, that dimers composed of catechin/epicatechin and catechin/catechin constituent units gave similar ^{13}C NMR chemical shifts for C-2, C-3, and C-4 of rings C and F in their peracetylated forms, but not in their free phenolic form. Thus, ^{13}C NMR data are needed for a final proof from the underivatized dimer from *Stryphnodendron adstringens*.

Proanthocyanidins can adopt different conformations with either quasi-equatorial or quasi-axial orientations of the B and E rings in the upper and lower flavan-3-ol units. Moreover, rotational isomers about the interflavanyl linkage afford compact or extended structures.[18,20,24,39,40] In compact conformers the E ring is positioned behind the A and C ring plane while in the extended conformers the E ring of the lower unit protrudes away from the A and C ring plane. To gain more information of the 3D structures of the compounds found here a conformational search for low energy conformers (LEC) has been undertaken using molecular mechanics (MM+) force-field calculations as described in the Experimental Section.

A conformational study of **1** resulted in two LECs in the compact form (Table 3). LECs with both rings B and E in quasi-equatorial (eq) orientations dominate. Comparison of the estimated and the experimentally measured $J_{2,3}$ and $J_{3,4}$ values revealed the best fit with the eq/eq conformer which is in accordance with the literature for catechin and epicatechin in which the E-conformation is strongly favored through the necessity to minimize 1,3-diaxial interactions and the pseudoallylic or A(1,3)-strain effect.[41] The quasi-axial C-3F galloyl moiety was observed to have a preferential alignment parallel to ring B instead of ring E. The predominance of a quasi-equatorial orientation of the B and E rings may also further be explained by the orientation of the galloyl group resulting in a $\pi-\pi$ stacking effect that contributes to the general stability of the conformation.[24] This allignment causes a steric hindrance with the quasi-axial flipping of the E ring (Figure 1). The heterocyclic ring of the lower unit adopts a clear half-chair conformation, whereas that of the upper unit shows a conformation between a C(2)-sofa and half-chair. Moreover, molecular mechanics calculations revealed a predominance of one rotamer in the compact form with a positive value of the C(3)−C(4)−D(8)−D(9) dihedral angle. Clearly separated signals for this rotamer were also observed in the ^{1}H and ^{13}C NMR spectra measured at 248 K, whereas ^{1}H NMR signals represented averaged broad proton signals and only a few signals in the ^{13}C NMR spectrum at room temperature due to free rotation of the subunits.

MM$^+$ calculations of **2** and **3** gave eq/eq, ax/eq, and eq/ax dimers (Table 3). The experimentally measured $J_{2,3}$ and $J_{3,4}$ values of **2** and **3** agreed best with the estimated ones of the eq/eq conformer in both cases (Table 3). Again, calculations demonstrated preference for the compact rotamer with the C ring between a C(2)-sofa and half-chair and the F ring in a half-chair conformation in which the B and E aryl groups are in quasi-equatorial orientations. Hence, **2** and **3** occur in conformations (Figure 1) that are similar to the conformation reported for procyanidin B-1, an epicatechin-(4β→8)-catechin.[24]

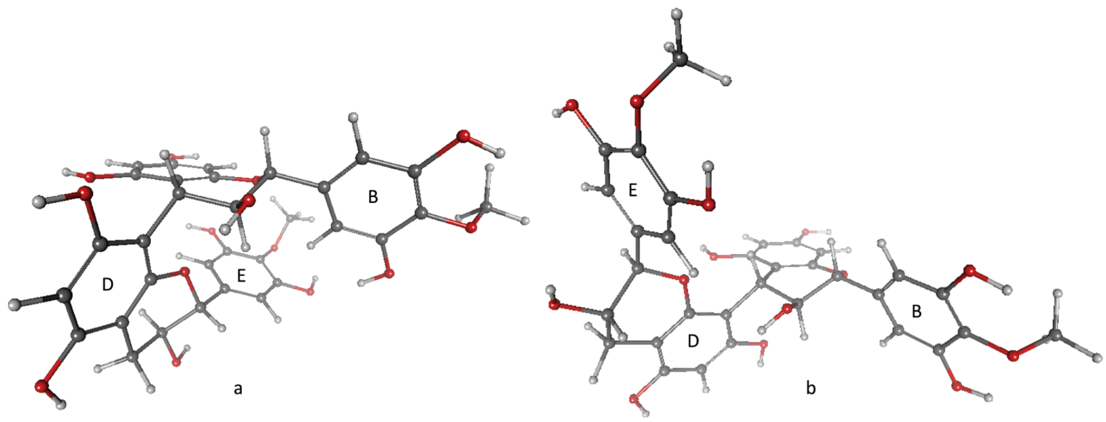

Figure 2. Energy-minimized structures proposed for **4** with the rings B and E in quasi-equatorial orientation and rotamers in (a) compact conformation [ϕ = C(3)−C(4)−D(8)−D(9) = negative] and (b) extended conformation [ϕ C(3)−C(4)−D(8)−D(9) = positive].

Signals for two rotamers were observed in the NMR spectra of **4**. Consequently, MM^{+} calculation also revealed the prevalence of two conformers, a compact and an extended structure, each with eq/eq, ax/eq, eq/ax, and ax/ax dimers (see Table 3). In the compact rotamers (ϕ = negative), the E and F rings of the lower unit were folded back under the plane of the upper unit (A and C rings). In the extended rotamers (ϕ = positive), the terminal unit was turned out of the plane of the upper unit. Analyses of the rotation energy at the interflavanyl linkage of the compact and extended conformers using PCModel, exhibited two energy-minimized states which differ by approximately 180° (data not shown). The hindered rotation may also be due to the steric interaction between the C-7D hydroxy group and those from either 3C and 5A.[42]

Estimated coupling constants matched best the experimental values for each rotamer when the two *O*-methylated pyrogallol B and E rings adopt a quasi-equatorial orientation, respectively. However, discrepancies between estimated (9.3 and 9.4 Hz) and measured (6.4 and 6.7 Hz) $J_{2,3}$ values in the lower units were observed which may suggest a significant proportion of a quasi-axial orientated conformer of the lower units. These $J_{2,3}$ values can be assumed as time-averaged molar ratios of a fast flipping between eq and ax conformers.[21,39,40,43] Interestingly, the smaller coupling constants for the terminal unit have been recently explained by the observation that the heterocyclic ring (F) takes a conformation between a half-chair and a skewed-boat, whereas the C ring exists in a half-chair conformation.[44] These conformations are also described and supported by NOESY experiments for procyanidin B-3, catechin-(4α→8)-catechin.[18] Collectively, both possibilities may contribute to the observed discrepancies in the coupling constants. It is well-known that a mixture of similar conformers exist in solution, whereas calculations consider only one conformation. Calculated conformations of the two rotamers of **4** which were similar not only to procyanidin B-3 but also to B-4[24] are shown in Figure 2.

The isolated proanthocyanidins were quantified in the ethanolic extract from the bark of *P. rigida* by HPLC analyses using a calibration curve with the respective isolated compound as previously described[1] to afford 1.9, 1.2, 4.2, and 1.4% for **1**, **2**, **3**, and **4**, respectively.

Preparations from *P. rigida* are used in traditional medicine for their wound-healing properties. To gain insight whether the isolated compounds also contribute to this effect, **2**−**4** were studied in a scratch assay. This assay affords details of the migration to and proliferation into an artificial wounded monolayer of Swiss 3T3 mouse fibroblasts.[45] Platelet-derived growth factor (PDGF) was used as a positive control at 2 ng/mL and showed an average 59.5% stimulating effect. All isolated compounds showed enhanced cell numbers at 1 μM concentration with compound **4** being the most active. Higher concentrations mostly led to a reduced activity, (Figure 3) which may be partially explained by possible cytotoxic effects on 3T3 fibroblasts. Antiproliferative effects have been described for some proanthocyanidins, such as prodelphinidin B-1 and B-2 in various cancer cell lines[46] and for a mixture of procyanidins and monomeric catechins in 3T3 fibroblasts.[47] Further studies need to be undertaken to elucidate whether the known antioxidative properties of procyanidins[9] are involved in the wound-healing effects. Interestingly, it has already been shown that a grape seed proanthocyanidin extract upregulated both hydrogen peroxide as well as TNF-α-induced VEGF expression and release contributing to wound-healing effects.[48,49]

The complex pathophysiological process of wound-healing also includes initial inflammatory processes.[50] However, delayed wound-healing may be observed if this process gets out of control. To investigate the inhibitory influence on inflammatory processes, compound **2** was studied in the NF-κB electrophoretic mobility shift assay. NF-κB is a central protein regulating the transcription of many inflammatory and proinflammatory cytokines and enzymes. Its inhibition by dimeric procyanidins was demonstrated in Jurkat cells.[51,52] Compound **2** only moderately impaired TNF-α-induced NF-κB after 24 h of incubation (Figure 1S in Supporting Information), which was only slightly influenced by cytotoxic effects (see Supporting Information). Therefore, proanthocyanidins seem to influence NF-κB only moderately.

We succeeded in the structure elucidation of four proanthocyanidins isolated from the ethanolic extract of the bark of *Parapiptadenia rigida*, three of which are described here for the first time. The conformational search combined with the NMR data confirmed the compact conformation with the bulky groups at C-2 in a quasi-equatorial orientation as the preferential arrangement in all cases. Presumably compact conformation minimizes the surface area of the molecule and hence solute−solvent contact.[40] As these prodelphinidins showed similar conformational results to those widely studied procyanidins, it

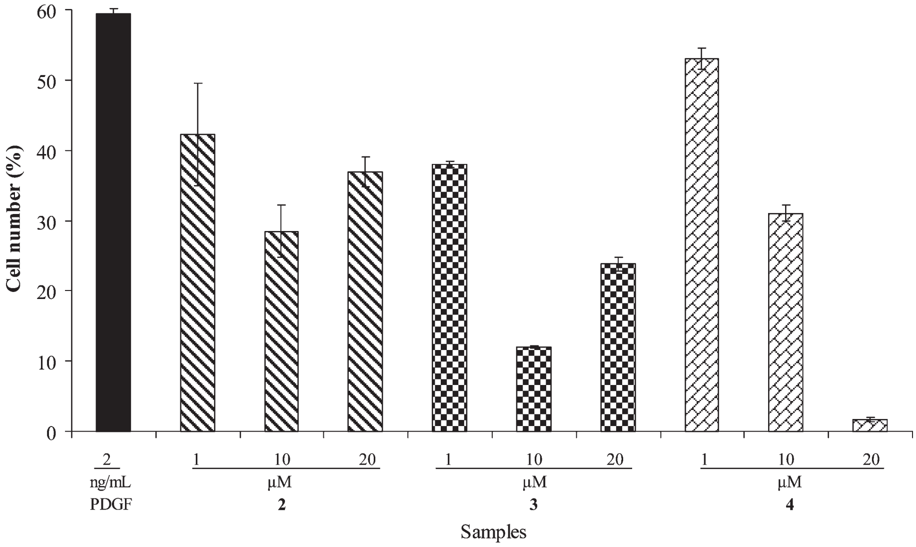

Figure 3. Effect of the isolated proanthocyanidins on the migratory and proliferative activities of 3T3 Swiss fibroblasts in the scratch assay after 12 h of incubation (37 °C; 5% CO_2). Positive control: PDGF (2 ng/mL); isolated compounds at 1, 10, and 20 μM, **2** (epigallocatechin-(4β→8)-4′-*O*-methylgallocatechin); **3** (epicatechin-(4β→8)-4′-*O*-methylgallocatechin); **4** (4α→8)-bis-4′-*O*-methylgallocatechin). Data are expressed as % of cells that migrate and proliferate to the wounded area compared to the negative control. Bars represent means ± SEM of three experiments.

can be assumed that the hydroxylation pattern of the catechol ring has no influence on the conformational behavior.

Moreover, phytochemical studies of *P. rigida* and the biological data suggested catechin derivatives also belong to the compounds benefitting the reepithelialization phases of the wound-repair process. Further studies should be performed to confirm these effects on reepithelialization *in vivo*.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded in MeOH at 20 °C on a Perkin-Elmer polarimeter, model 341, CD spectra on a Jasco J-715 spectropolarimeter, at 200−350 nm in MeOH, and IR spectra on a Perkin-Elmer Spectrum One FT-IR spectrometer with ATR sampling. NMR spectra were recorded in methanol-d_4 on a Bruker DRX instrument at 400 MHz (^{1}H) and 100 MHz (^{13}C). MS data were taken with the following instruments: APCI/ESIMS, LCQ-Advantage mass spectrometer (Thermo Fisher); HR-ESIMS, LTQ Orbitrap XL mass spectrometer (Thermo Fisher); HR-EIMS, MAT-95XL double-focusing magnetic field mass spectrometer (Thermo Fisher). MPLC was carried out with Eurosil Bioselect 100, C-18 (20−45 μm) and open-column chromatography with Sephadex LH-20. Column fractions were monitored by TLC (silica gel 60 F 254, Merck) and detection was done at 254 and 366 nm and with anisaldehyde-H_2SO_4 acid and heating at 110 °C. Analytical TLC was carried out with an Automatic TLC Sampler (CAMAG). HPLC analysis was performed on a Hewlett-Packard 1090 apparatus, using a Phenomenex Luna C-18 column (150 × 4.6 mm, 3 μm) with mobile phases A (H_2O/MeCN − 95:5) and B (MeOH/H_2O − 95:5), both with 0.1% HCOOH. Linear gradient starting with 5% of B, increasing to 30% at 30 min, 50% at 45 min, and 100% from 50 to 55 min; re-equilibration of the column from 56 to 65 min, flow rate 0.5 mL/min, detection at 275 nm; sample injection of 20 μL.

Plant Material. The bark from *Parapiptadenia rigida* (Benth.) Brenan was collected from the natural habitat of the plants located on "Morro Cechela" in Santa Maria, Rio Grande do Sul, Brazil in October 2007 and was identified by Dr. Solon Jonas Longhi, Federal University of Santa Maria - UFSM. A voucher specimen was deposited at the herbarium of the University, code SMDB 12309.

Extraction and Isolation. Air-dried and powdered bark (1.3 kg) was extracted with EtOH using a Soxhlet apparatus. The crude ethanolic extract was concentrated under vacuum at 40 °C to yield 230.2 g of extract, which was treated with MeOH at −20 °C, giving a soluble part of 221.4 g after solvent removal. Initial fractionation of 6 g of the ethanolic extract was carried out using open-column liquid chromatography on Sephadex LH-20 (60 × 6 cm) with MeOH and yielded 14 fractions. Fraction 6 (193 mg) was subfractionated by MPLC with RP-18 silica gel (50 × 1.2 cm) using a flow rate of 0.7 mL/min and mixtures of MeOH−H_2O (30−100%) to afford **3** (50.0 mg). Fraction 7 (326 mg) was rechromatographed by MPLC with H_2O/MeOH/MeCN (80:15:5) at a flow rate of 0.8 mL/min, yielding 9 subfractions, from which fractions 2 and 7 gave **2** (11.5 mg) and **4** (7.0 mg), respectively. Fraction 12 was separated by MPLC at a flow rate of 0.7 mL/min and mixtures of MeOH−H_2O (20 - 70%) to obtain **1** (40.8 mg).

Cell Culture. Jurkat T cells (ACC No 282) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL).

Swiss 3T3 albino mouse fibroblasts (Cell Line Service, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin and maintained at 37 °C in a humidified, 5% CO_2 environment (Gibco-BRL).

Scratch Assay. Wound-healing properties were evaluated in vitro using Swiss 3T3 albino mouse fibroblasts and a scratch assay as previously described.[45,53] The isolated compounds were tested at 1, 10, and 20 μM concentrations. PDGF (2 ng/mL) was used as a positive control. A negative control containing only cells and 4 μL of DMSO was used as a reference to calculate the percentage rate of the increase in cell number for each sample after 12 h incubation. The experiments were performed in triplicate. The data were analyzed using CellC software.[54]

NF-κB Electrophoretic Mobility Shift Assay. Jurkat T cells (3×10^5 cells/mL) were preincubated with the isolated compounds (10, 20, 30, 55 μM) for 24 h and subsequently stimulated for 1 h with rh-TNF-α at 2.5 ng/mL (R&D systems). Nuclear cell extracts were prepared as previously described.[51] NF-κB oligonucleotide (Promega) was labeled using [γ-^{33}P] dATP (3000 Ci/mmol; Amersham). The specificity of the NF-κB-DNA binding was assessed by competition with a 100-fold molar excess of unlabeled oligonucleotide containing the consensus sequence for NF-κB. The bands were quantified densitometrically using a PhosphoImager scan.

MTT Assay. Cytotoxic activity was studied using the MTT colorimetric assay as previously described and modified to 96-well plate.[55] Detailed information is given in the Supporting Information.

Statistical Analysis. Statistical analyses were carried out using the Origin Scientific Graphing and Analysis Software, version 7.0 or Microsoft Office Excel 2007. Data are expressed as the mean ± SEM.

Conformational Analysis. Computational search for low-energy conformers was performed using molecular mechanics force field calculations MM+ from the molecular modeling software Hyperchem (v. 6.02). The structures were minimized to a final root-mean-square (rms) value gradient of 0.01 kcal mol^{-1} $Å^{-1}$ and 1000 cycles. The search was limited to 100 energy-minimized conformers for each dimer. These 100 conformers were grouped using the function rms fit and overlay according to the following two criteria: (i) the orientation of rings B and E in quasi-eq or quasi-ax related to the heterocyclic ring conformation; (ii) the conformation at the interflavan linkage (compact or extended rotamers). Finally, the lowest energy-minimized conformer (LEC) of each group was selected and their $J_{2,3}$ and $J_{3,4}$ values were estimated. This estimation was done by the H(2)−C(2)−C(3)−H(3) and H(3)−C(3)−C(4)−H(4) dihedral angles (ϕ) using the Karplus equation for vicinal protons[56] updated by Aydin and co-workers,[57] respectively. The interflavan linkage conformation was established by measuring the C(3)−C(4)−D(8)−D(9) dihedral angle (ϕ) of each LEC. The estimated coupling constants (J) were compared with the experimentally obtained ones to find the best suitable conformation.

Epigallocatechin-(4β→8)-epigallocatechin-3-O-gallate (***1***): brownish, amorphous powder; CD (MeOH) $\Delta\varepsilon_{205}$ (−7.2), $\Delta\varepsilon_{225}$ (+16.7), $\Delta\varepsilon_{233}$ (+15.9), $\Delta\varepsilon_{273}$ (−1.2), $\Delta\varepsilon_{296}$ (+0.9); $[\alpha]^{20}_{D}$ +28 (*c* 1.0, MeOH); IR (neat) ν_{max} 3362, 1606, 1519, 1444, 1318, 1197, 1142, 1097, 1033, 819, 730 cm^{-1}; ^{1}H NMR (methanol-d_4,400 MHz) δ 2.91 (dd, 2.5, 17.1, H-4Fβ), 3.08 (dd, 4.4, 17.1, H-4Fα), 3.9 (brs, H-3C), 4.81 (brs, H-4C), 5.09 (brs, H-2C), 5.15 (brs, H-2F), 5.6 (m, H-3F), 5.91 (s, H-6D), 5.97 (d, 1.6, H-6A), 6.01 (d, 1.6, H-8A), 6.41 (s, H-2′B/H-6′B), 6.62 (s, H-2′E/H-6′E), 7.04 (s, H-2′′/6′′); ^{13}C NMR (methanol-d_4, 100 MHz) δ 166.4 (C-7′′), 156.9 (C-7A), 156.5 (C-5A), 156.4 (C-9A), 155.2 (C-7D), 154.5 (C-5D), 153 (C-9D), 145.2 (C-3′B/E, C-5′B/E), 144.7 (C-3′′/5′′), 138.4 (C-4′′), 132.2 (C-4′E), 131.9 (C-4′B), 130.5 (C-1′B), 129.3 (C-1′E), 119.9 (C-1′′), 108.9 (C-2′′/6′′), 106.7 (C-8D), 105.1 (C-2′B/6′B), 105 (C-2′E/6′E), 100.7 (C-10A), 98 (C-10D), 95.6 (C-6D), 94.8 (C-6A), 94.2 (C-8A), 76.8 (C-2F), 75.8 (C-2C), 72.2 (C-3C), 67.9 (C-3F), 35.4 (C-4C), 25.4 (C-4F); ESIMS (negative mode) m/z 761 $[M-H]^-$ (100); (positive mode) m/z 763 $[M+H]^+$ (100).

Epigallocatechin-(4β→8)-4′-O-methylgallocatechin (***2***): brownish, amorphous powder; CD (MeOH) $\Delta\varepsilon_{207}$ (−6.6), $\Delta\varepsilon_{217}$ (+18.6), $\Delta\varepsilon_{233}$ (+7.3), $\Delta\varepsilon_{290}$ (+1.5); $[\alpha]^{20}_{D}$ +23 (*c* 2.0, MeOH); IR (neat) ν_{max} 3303, 1605, 1515, 1449, 1348, 1193, 1144, 1103, 1043, 1017, 821, 752, 703 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1; HRESIMS m/z 625.15503 (calcd for $C_{31}H_{28}O_{14}$ + H, 625.1557); ESIMS (negative mode) m/z 623 $[M-H]^-$ (100); (positive mode) m/z 625 $[M+H]^+$ (100).

Epicatechin-(4β→8)-4′-O-methylgallocatechin (***3***): brownish, amorphous powder; CD (MeOH) $\Delta\varepsilon_{206}$ (−4.5), $\Delta\varepsilon_{216}$ (+13.2), $\Delta\varepsilon_{233}$ (+6.5), $\Delta\varepsilon_{289}$ (+1.7); $[\alpha]^{20}_{D}$ +14 (*c* 1.0, MeOH); IR (neat) ν_{max} 3351, 1607, 1519, 1446, 1355, 1202, 1143, 1052, 675 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 1; HRESIMS m/z 609.1603 (calcd for $C_{31}H_{28}O_{13}$ + H, 609.1608); ESIMS (negative mode) m/z 607 $[M-H]^-$ (100); (positive mode) m/z 609 $[M+H]^+$ (100).

(4α→8)-Bis-4′-O-methylgallocatechin (***4***): brownish, amorphous powder; CD (MeOH): $\Delta\varepsilon_{204}$ (+6.5), $\Delta\varepsilon_{214}$ (−29.6), $\Delta\varepsilon_{236}$ (−8.2), $\Delta\varepsilon_{269}$ (+1.3); $[\alpha]^{20}_{D}$ −104 (*c* 1.3, MeOH); IR (neat) ν_{max} 3211, 1604, 1447, 1355, 1143, 1052 cm^{-1}; ^{1}H and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 2; HRESIMS m/z 639.1702 (calcd for $C_{32}H_{30}O_{14}$ + H, 639.1708); ESIMS (negative mode) m/z 637 $[M-H]^-$ (5); (positive mode) m/z 662 $[M+Na]^+$ (44), 639 $[M+H]^+$ (86), 457 (11). APCIMS (negative mode) m/z 637 $[M-H]^-$ (100), 319 (14); (positive mode) m/z 639 $[M+H]^+$ (100), 321 (75).

ASSOCIATED CONTENT

Supporting Information. The ^{1}H, ^{13}C, HSQC, HMBC, and COSY spectra of **2** to **4**, as well as the results from the NF-κB EMSA and the MTT assay of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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