Phenolic Metabolites of *Dalea versicolor* that Enhance Antibiotic Activity against Model Pathogenic Bacteria

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A new flavonoid (**1**) was isolated from organic extracts of *Dalea versicolor*, along with six known phenolic compounds (**2**−**7**). The structures of the seven compounds were determined by NMR and HRMS methods. These compounds were evaluated for direct activity against a variety of organisms in vitro, including the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus*. In addition, the compounds were evaluated for their ability to potentiate the activity of known antimicrobials through inhibition of multidrug-resistance (MDR) pumps. Compounds **1**, **2**, **4**, and **7** exhibited direct or synergistic activity toward the human pathogen *S. aureus* and the opportunistic pathogen *B. cereus*. Compounds **4** and **7** were also found to potentiate the activity of berberine and of prescribed antibiotics, with **4** demonstrating a mode of action consistent with inhibition of the NorA MDR efflux pump in *S. aureus*.

There is a growing body of evidence to suggest that phenolic compounds, like flavonoids, have the ability to inhibit multidrug-resistance (MDR) pumps in bacteria. Flavonoids with direct activity against methicillin-resistant *Staphylococcus aureus* have been recently reported.[1] In addition, detailed studies of flavonoids and other compounds, and their likely inhibition of NorA, a transmembrane protein that functions as an antibiotic "efflux pump", in *S. aureus* have been carried out.[2−4]

Dalea spp. such as *D. scandens* var. *paucifolia* and *D. purpurea* have been shown to contain flavonoids with antimicrobial activity.[1,5] Our continuing work on the genus *Dalea* has led to the investigation of compounds from *Dalea versicolor* Zucc. var. *sessilis* (A. Gray) Barneby 'mountain delight' (Fabaceae), a shrub native to the Sonoran Deserts of Arizona and Mexico. To our knowledge, *D. versicolor* has never been studied in detail for its content of secondary metabolites. A closely related species, *D. formosa*, was used by Pueblo and Apache Indians for analgesic purposes, and by Hopi for influenza and other viral infections.[6]

Organic extracts of *D. versicolor*, both alone and in the presence of berberine, a relatively weak antibiotic, exhibited strong activity (MIC = 7.8 μg/mL) against the human pathogen *S. aureus*. Fractionation of these extracts afforded a new prenylated isoflavanone (**1**) and six known compounds, including two prenylated flavanones (**2** and **3)**, a chalcone (**4**), a pterocarpin (**5**), and two stilbenes (**6** and **7**). The structures of all seven compounds were determined by NMR and HRMS techniques. Details of the isolation, structure determination, and inhibitory antibiotic activities of these metabolites are reported here.

Fractionation of organic extracts of *D. versicolor* var. *sessilis* by silica gel VLC, Sephadex LH-20, and repeated normal- and reversed-phase chromatography over silica gel afforded metabolites **1**−**7**.

The HRESIMS, ^{13}C NMR (Table 1), and DEPT data for compound **1** indicated a molecular formula of $C_{25}H_{28}O_6$. Four methyl singlets were present in the ^{1}H NMR spectrum (Table 1), and HMBC correlations between these hydrogens and the carbons of their respective isoprene-derived units ultimately accounted for 10 carbons and 18 hydrogens. ^{13}C and DEPT experiments indicated the presence of 12 quaternary carbons, including a ketone carbonyl at δ 197.6 and five oxygenated sp^2 carbons between δ 155 and 165. These data, along with the remaining structural fragments suggested a highly oxygenated, substituted aromatic ring system. The overall pattern of ^{13}C NMR chemical shift values indicated a flavonoid structure, which was confirmed by HMQC and HMBC spectral data and by comparison with known compounds.[1,7,8] Chemical shift values for the methylene at C-2 and the methine at C-3 (δ 69.9 and 45.6, respectively) were typical of an isoflavanone structure.[9] Key HMBC correlations were observed between H-6′ and C-2′, C-4′, C-1‴, and C-3, which established

Table 1. NMR Data for Compound **1** in $CDCl_3$

position	δ_C[a]	δ_H[b] (mult.; J_{HH})	HMBC (^{1}H → ^{13}C)
2	69.9	4.91 (dd; 12.0, 4.9)	C-3, 4, 9, 1′
		4.72 (dd; 12.0; 4.9)	C-3, 4, 9, 1′
3	45.6	4.00 (br t; 6.0)	C-2, 4, 1′, 2′, 6′
4	197.6		
5	163.2		
5-OH			C-5, 6, 10
6	97.3	6.00 (s)	C-5, 7, 8, 10
7	164.5		
8	106.7		
9	160.0		
10	102.2		
1′	114.5		
2′	155.1		
3′	107.0	6.46 (s)	C-1′, 2′, 4′, 5′
4′	155.9		
5′	125.4		
6′	125.6	7.31 (s)	C-3, 2′, 4′, 1‴
1″	21.8	3.33 (d; 7.0)	C-7, 8, 9, 2″, 3″
2″	121.8	5.19 (br t; 7.5)	
3″	134.9		
4″	26.0	1.75 (s)	C-2″, 3″, 5″
5″	18.1	1.81 (s)	C-2″, 3″, 4″
1‴	40.1		
2‴	148.2	6.16 (dd; 17.6, 10.3)	C-1‴, 4‴, 5‴
3‴	113.8	5.35*cis* (d; 17.6)	C-1‴, 2‴
		5.30*trans* (d; 10.3)	C-1‴, 2‴
4‴	27.3	1.41 (s)	C-5′, 1‴, 2‴, 5‴
5‴	27.3	1.39 (s)	C-5′, 1‴, 2‴, 4‴

[a] 75 MHz. [b] 300 MHz.

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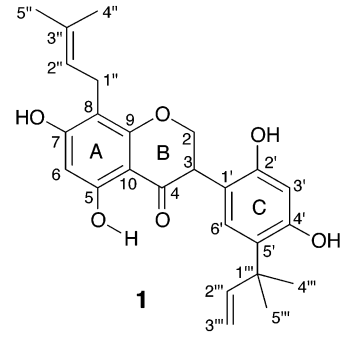

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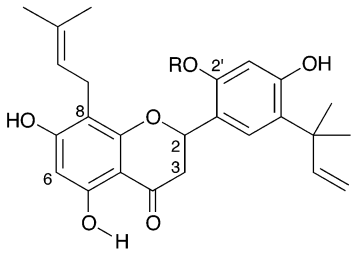

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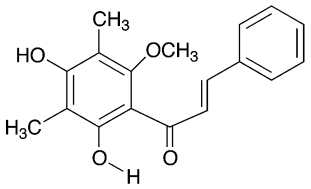

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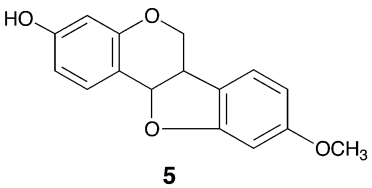

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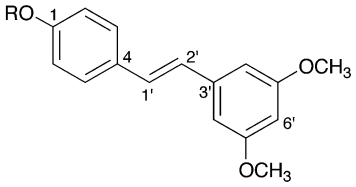

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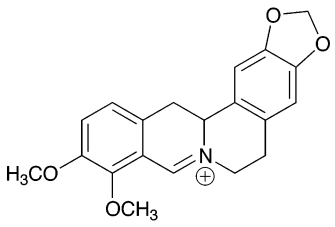

berberine

connectivity between the B and C rings and helped place the dimethylallyl side chain at C-5′. Similarly, the substitution pattern of the C ring was further confirmed by correlations between H-3 and C-1′, C-2′, and C-6′. Correlations from H$_2$-2 to C-9 and C-1′ helped confirm the connectivity between rings A, B, and C. The position of the prenyl side chain at C-8 was established with correlations between H$_2$-1″ and C-7, C-8, and C-9. The hydrogen-bonded phenolic OH at δ 11.80 in the ^{1}H NMR spectrum was assigned to C-5 and showed HMBC correlations to C-5, C-6, and C-10, thus finalizing the substitution pattern of the A ring of dalversinol A (**1**) as shown. To our knowledge, the closest related isoflavanone to **1** is tomentosanol A,[7] which is prenylated at C-6, rather than at C-8, and is methoxylated at C-2′.

Table 2. Susceptibility of *S. aureus*, NorA, and *B. cereus* in Antimicrobial Agents without and with Compounds **4** and **7**

	S. aureus (MIC μg/mL)		NorA mutant *S. aureus* (MIC μg/mL)		*B. cereus* (MIC μg/mL)		
	alone	+**4**[a]	alone	+**4**[a]	alone	+**4**[a]	+**7**[a]
berberine	500	31.25	64	16	125	3.91	7.81
erythromycin	0.5	0.1	0.1	<0.1	0.4	0.1	0.2
tetracycline	0.98	0.18	0.4	0.1	1	0.2	0.5

[a] **4** and **7** were added at a final concentration of 10 μg/mL.

The HRESIMS of **2** revealed a molecular formula of $C_{25}H_{28}O_6$. The ^{13}C and DEPT data for **2** revealed multiplicity and chemical shift values for C-2 and C-3 (methine at δ 77.1 and methylene at δ 42.2, respectively) that, in contrast to **1**, were characteristic of a flavanone structure.[7,8] The ^{1}H and ^{13}C NMR data for **2** were nearly identical to published values for two known compounds that differed only in the placement of a prenyl group at C-6 or C-8.[1,8] The position of the prenyl group at C-8 in **2** was confirmed, as in **1** above, by HMBC correlations between H$_2$-1″ and C-7, C-8, and C-9. Spectral data for compound **3** were nearly identical to that of **2**, with the addition of a methoxy carbon (δ 55.7) and protons (δ 3.80, singlet). An HMBC correlation between the methoxy protons and C-2′, along with comparison to the NMR assignments for **2**, led to placement of this group. HMBC correlations, and agreement with published values,[1] were sufficient to assign the overall structures of the known compounds **2** and **3** as shown.

Interpretation of NMR spectroscopic data led to the structure of compound **4**, a known chalcone,[10] with the molecular formula $C_{18}H_{18}O_4$ as confirmed by HRESIMS. Complete spectral data for **4** (see Experimental Section) has been previously reported only for its diacetate derivative. The structures of known compounds **5**−**7** were determined by NMR spectroscopic and mass spectrometric methods, and these data corresponded in all essential respects with those reported in the literature.[11−16]

Compounds **1**−**7** were evaluated in antimicrobial assays in order to determine their activity both individually and in synergy with known antibiotics. The compounds were tested individually against the human pathogen *S. aureus*. Compounds **1** and **2** exhibited direct activity, with MICs of 31.3 and 7.8 μg/mL, respectively. Compound **4** in particular, but also compound **7**, demonstrated the greatest differential activity related to MDR inhibition. Both were very weakly active alone (MICs of 250 and 500 μg/mL, respectively), but they caused complete growth inhibition at very low concentrations (~3.3 μg/mL) in combination with a subinhibitory concentration of berberine. Furthermore, both compounds exhibited a similar level of action against the opportunistic pathogen *B. cereus*, when tested alone and in the presence of berberine. Chalcone **4** was evaluated in further detail alone and in combination with three known antibiotics, as shown in Table 2. The compounds were tested against *S. aureus* and an *S. aureus* NorA knock out mutant that lacks the NorA efflux pump.[17] As expected, the activity of all of the compounds increased

against the more susceptible NorA mutant *S. aureus* when compared to the wild-type *S. aureus*. The addition of chalcone **4** increased the activity of the antibiotics in each case, but did so to a lesser extent in the case of the NorA *S. aureus* mutant, indicating a likely mode of action associated with the NorA pump. Compounds **4** and **7** were also tested against *B. cereus* in a similar manner, as shown in Table 2. Both compounds were able to potentiate the activity of known antibiotics. In particular, compound **4**, in the presence of berberine, effected a dramatic 30-fold increase in activity against *B. cereus*, an organism that is virtually identical with *B. anthracis*, the bacterium causing anthrax.[18]

This is the first known case where compounds with strong direct antimicrobial activity and those with MDR-pump inhibitory action have been demonstrated to be present in the same plant. It is likely that the overall antibiotic activity observed in the crude *D. versicolor* extract is due to the presence of both.

Experimental Section

General Experimental Procedures. NMR spectra were acquired on a Varian Unity Inova 400 spectrometer, equipped with an inverse detection probe, and on a Varian Unity Plus 300 spectrometer. Optical rotations were obtained on a Jasco model P-1010 polarimeter. IR spectra were recorded on a Nicolet Avatar 360 FT-IR spectrophotometer, and UV spectra were acquired on a Hewlett-Packard 8453 diode array spectrophotometer. ESIMS/MS and HRESIMS were obtained in positive ion mode on a Micromass Q-TOF mass spectrometer. EIMS was obtained on a Shimadzu GCMS-QP5000 equipped with a DI-50 direct sample inlet device.

Plant Material. Whole plants of *Dalea versicolor* Zucc. var. *sessilis* (A. Gray) Barneby (Fabaceae) 'mountain delight' were collected by G.N.B. and Frank Stermitz in March 1998 from Starr Nursery, Broadway Ave., Tucson, AZ. A second, larger collection was made by Lt.Col Michael P. Lynch, Arizona National Guard, in April 2001 from Summer Winds Nursery, 17826 N. Tatum Blvd., Phoenix, AZ. A voucher specimen (#83554) was authenticated by J. Ackerfield, Department of Biology, Colorado State University, and is deposited in the Colorado State University Herbarium. Plants were air-dried for several days and then stored in a −20 °C freezer prior to extraction.

Cell Culturing and Susceptibility Testing. *S. aureus* and *B. cereus* were cultured in Mueller-Hinton (MH) broth. Cells (10^5/mL) were inoculated into MH or YPD broth respectively and dispensed at 0.2 mL/well in 96-well microtiter plates. The rationale to detect MDR inhibitory activity was to test the combined action of a plant extract with an antimicrobial added at a subinhibitory concentration. Extracts from *D. versicolor* that inhibited cell growth in the presence of the antimicrobial and had no activity when added alone were likely to contain a compound with the potential to act as an MDR inhibitor.

Bioassay-guided fractionation was used initially to detect MDR inhibitors. Growth inhibition was determined by serial 2-fold dilution of test fraction/compounds accompanying 30 μg/mL berberine against *S. aureus* and *B. cereus*, 10 μg/mL erythromycin, and tetracycline. A likely MDR inhibitor was defined as a compound that completely prevented cell growth in the presence of subinihibitory antibiotic during an 18 h incubation at 37 °C (*S. aureus*) or 24 h at 30 °C (*B. cereus*). All tests were done in triplicate by following National Center for Clinical Laboratory Standards recommendations (NCCLS).[19] Growth was assayed with a microtiter plate reader (Spectramax PLUS384, Molecular Devices) by absorption at 600 nm.

Extraction and Isolation. Plant material (72 g) was extracted successively with EtOAc and MeOH (1 L, 24 h each) to provide, after evaporation, 4.4 g of crude combined extract. This material was preadsorbed in MeOH solution onto ~10 g of silica gel, the solvent removed under vacuum, and the resulting powder subjected to vacuum liquid chromatography (VLC) over a prepacked column bed, 6 cm (i.d.) × 3 cm (h), of TLC-grade silica gel (Selecto Scientific). The column was eluted using a stepwise gradient of solvents (200 mL each), beginning with hexane and continuing with mixtures of EtOAc in hexane (20%, 40%, 60%, 80%, 100%), followed by mixtures of MeOH in CH_2Cl_2, up to 30%. The four fractions that eluted with 20−80% EtOAc were combined on the basis of TLC analysis (EM Science, silica gel 60, F_{254}, with H_2SO_4/vanillin spray reagent), and the solvents were evaporated. The residue (1.6 g) was further fractionated by Sephadex LH-20 (Sigma) column chromatography (2.5 cm × 23 cm) eluting with 1 L of 3:1:1 hexane−toluene−MeOH at a flow rate of 0.4 mL/min, and collecting ~5 mL fractions. Fractions of similar composition as determined by TLC were pooled, resulting in 14 fractions. Fraction 6 (71.5 mg) from this column was further purified over silica gel (1.2 cm × 6.4 cm, Davison Chemical 100−200 mesh) by gravity elution using a step-gradient of 100% CH_2Cl_2, followed by MeOH in CH_2Cl_2 (0.5%, 1%, 2%, 5%, 10%, 30%; 50 mL fractions). Fraction 2 from this column afforded chalcone **4** (7 mg).

Fractions 7−9 (179 mg) from the Sephadex column were further purified over silica gel in a manner similar to that described above, resulting in a 111 mg fraction of interest (red spot w/spray reagent on TLC, $R_f = 0.8$, in 9:1 CH_2Cl_2−MeOH). This fraction was purified by repeated chromatography over C_{18} (H_2O−CH_3CN, Alltech Extract-Clean Column, 1000 mg) and over silica gel (Davison) eluting with increasing amounts of EtOAc in hexane to yield medicarpin (**5**, 5 mg).

The methanolic extract (20 g) of a second collection of plant material (108 g) was purified by silica gel VLC, and fractions eluting with 25−100% EtOAc in hexane were combined and triturated with hexane. The remaining solids were partitioned over Sephadex LH-20, using methods described earlier, resulting in 25 fractions. Fractions 6 and 17 from this column were further purified by silica gel column chromatography in CH_2Cl_2−MeOH, resulting in stilbenes **7** (19 mg) and **6** (10 mg), respectively. Fraction 20 from the Sephadex column was purified using a linear gradient of EtOAc (0−100%) in hexane (silica gel 100−200 mesh, flow rate ~20 mL/min). Fractions from this column that eluted with 20−25% EtOAc were combined to afford flavanone **2** (27 mg). Fractions 15 and 16 from the Sephadex column were each chromatographed using procedures similar to those described above, in successive steps over C_{18} in H_2O−CH_3CN (fractions of interest eluting at 60% CH_3CN), over silica gel in CH_2Cl_2−MeOH (2−5% MeOH fractions), and over silica gel in hexane−EtOAc (40% EtOAc fractions) to yield flavanone **3** (7 mg) and dalversinol A **1** (11 mg).

Dalversinol A (1): pale yellow solid; $[\alpha]_D$ −15.8° (*c* 0.17, $CHCl_3$); mp 86−92 °C; UV(MeOH) λ_{max} (log ϵ) 204 (4.22), 229 (3.83), 291 (3.71); IR ν_{max} ($CHCl_3$) 3300 (br OH), 3020, 2928, 1638, 1601, 1496, 1472, 1449, 1378, 1272, 1160 cm^{-1}; ^1H, ^{13}C, HMBC NMR data, see Table 1; HRESIMS found *m*/*z* 447.1837 $(M + Na)^+$, calcd for $C_{25}H_{28}O_6Na$ 447.1784.

2(*S*)-5′-(1‴,1‴-Dimethylallyl)-8-(3″,3″-dimethylallyl)-2′,4′,5,7-tetrahydroxyflavanone (2): yellow solid; $[\alpha]_D$ −76.8° (*c* 0.17, $CHCl_3$) [lit. −79.1° (*c* 5.7, $CHCl_3$)];[1] mp 110−120 °C (lit. 138−140 °C);[1] UV, ^1H and ^{13}C NMR consistent with literature values;[1] the structure of **2** was confirmed by HMQC and HMBC NMR spectroscopy; EIMS *m*/*z* 424 (M^+, rel int 4), 423 (13), 405 (56), 390 (9), 362 (58), 351 (37), 337 (14), 219 (17), 205 (30), 177 (45), 165 (100).

2(*S*)-5′-(1‴,1‴-Dimethylallyl)-8-(3″,3″-dimethylallyl)-2′-methoxy-4′,5,7-tetrahydroxyflavanone (3): yellow oil; $[\alpha]_D$ −46.5° (*c* 0.17, $CHCl_3$) [lit. −91.9° (*c* 5.3, $CHCl_3$)];[1] mp 76−84 °C (lit. 80−82 °C);[1] UV, ^1H and ^{13}C NMR consistent with literature values;[1] the structure of **3** was confirmed by HMQC and HMBC NMR spectroscopy; HRESIMS found *m*/*z* 461.1993 $(M + Na)^+$, calcd for $C_{26}H_{30}O_6Na$ 461.1940.

4′,6′-Dihydroxy-3′,5′-dimethyl-2′-methoxychalcone (4): yellow powder; $[\alpha]_D$ −7.0° (*c* 0.20, $CHCl_3$); mp 112−118 °C; UV(MeOH) λ_{max} (log ϵ) 205 (4.39), 340 (4.19); IR ν_{max} (film on NaCl) 3443 (br OH), 2926, 1628, 1559, 1495, 1449, 1350, 1166,

1111 cm^{-1}; ^1H NMR ($CDCl_3$) δ 13.63 (s, OH-6′), 8.02 (d, 15.8, H-α), 7.86 (d, 15.7, H-β), 7.67 (m, H-2, 6), 7.43 (m, H-3, 4, 5), 5.36 (br s, OH-4′), 3.69 (s, OCH_3-2′), 2.18 (s, CH_3-5′), 2.16 (s, CH_3-3′), ^{13}C NMR ($CDCl_3$) δ 193.6 (C═O), 162.3 (C-6′), [a]159.4 (C-4′), [a]159.2 (C-2′), 143.1 (C-β), 135.7 (C-1), 130.4 (C-4), 129.2 (C-3, 5), 128.7 (C-2, 6), 127.0 (C-α), [b]109.4 (C-1′), [b]109.1(C-3′), 106.8 (C-5′), 62.6 (OCH_3-2′), 8.5 (CH_3-5′), 7.8 (CH_3-3′), ([a,b]Assignments may be interchanged); HMBC correlations ($CDCl_3$) H-2 → C-4, 6, β; H-3,4,5 → C-2,6; H-6 → C-2,4,β; H-β → C-2,6,α,C═O; H-α → C-1,β,C═O; H_3CO → C-2′; H_3C-3′ → C-2′,3′,4′; H_3C-5′ → C-4′,5′,6′; HO-6′ → C-1′,5′,6′; HRESIMS found *m*/*z* 299.1245 $(M + H)^+$, calcd for $C_{18}H_{19}O_4$, 299.1283.

(+)-Medicarpin (5): white solid; $[\alpha]_D$ +113.9° (*c* 0.23, $CHCl_3$); mp 118−125 °C (lit. 127−129 °C); UV, ^1H and ^{13}C NMR consistent with literature values;[11−13] the structure of **5** was confirmed by HMQC and HMBC NMR spectroscopy; EIMS *m*/*z* 270 (M^+, rel int 100), 255 (36), 239 (4), 226 (5), 197 (7), 182 (6), 161 (15), 148 (32), 135 (29).

3,5-Dimethoxy-4′-hydroxy-*trans*-stilbene (6): pale yellow solid; $[\alpha]_D$ +2.4° (*c* 0.25, $CHCl_3$); mp 55−64 °C; UV (MeOH); λ_{max} (log ϵ) 218 (4.24), 239 sh (4.03), 306 (4.30), 321 (4.29); ^1H NMR consistent with literature values;[15] ^{13}C NMR ($CDCl_3$) δ 161.1 (C-5′, 7′), 155.5 (C-1), 139.9 (C-3′), 130.3 (C-4), 128.9 (C-1′), 128.2 (C-3, 5), 126.8 (C-2′), 115.8 (C-2, 6), 104.6 (C-4′, 8′), 99.8 (C-6′), 55.6 (CH_3O-5′, 7′); The structure of **6** was further confirmed by HMQC and HMBC NMR spectroscopy; EIMS *m*/*z* 256 (M^+, rel int 100), 240 (8), 224 (12), 210 (10), 195 (4), 181 (17), 165 (8), 152 (10), 141 (6).

3,5,4′-Trimethoxy-*trans*-stilbene (7): pale yellow solid; $[\alpha]_D$ +2.0° (*c* 0.19, $CHCl_3$); mp 42−44 °C (lit. 56−57 °C);[14] UV, ^1H and ^{13}C NMR consistent with literature values;[14−16] the structure of **7** was confirmed by HMQC and HMBC NMR spectroscopy; EIMS *m*/*z* 270 (M^+, rel int 100), 255 (6), 239 (16), 224 (11), 212 (6), 196 (9), 181 (6), 165 (7), 152 (11), 135 (15).

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