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AMORPHASTILBOL, AN ANTIMICROBIAL AGENT FROM *AMORPHA NANA*

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Key Word Index—*Amorpha nana*; Leguminosae; amorphastilbol; 3,5-dihydroxy-7,4′-dimethoxyflavone; antimicrobial agents; chemistry; bioactivity.

Abstract—Bioassay-directed fractionation of *Amorpha nana* demonstrated that the anti Gram positive/antitubercular constituent is amorphastilbol. The chemical structure of amorphastilbol was confirmed by chemical transformation to known (*Radula variabilis*) dihydroamorphastilbol and its methyl ether. Inactive 3,5-dihydroxy-7,4′-dimethoxyflavone was detected in this plant for the first time and identified by interconversion with kaempferol. All compounds were evaluated *in vitro* for antimicrobial spectrum and potency.

INTRODUCTION

In a previous study we demonstrated that the antimicrobially active constituents of *Amorpha fruticosa* were the acidic bibenzyls amorfrutin A and B [1] and the rotenoid 11-hydroxytephrosin [2]. Subsequently, we detected antimicrobial activity in extracts of the leaves of the related species *A. nana* from North Dakota. Chromatographic examination suggested that the responsible agent(s) was(were) different than those in *A. fruticosa* so the fractionation study reported herein was undertaken.

RESULTS AND DISCUSSION

A single active agent was isolated by chromatography from the polar lipid fraction and was readily demonstrated to be the known *A. nana* constituent amorphastilbol (**1**) [3]. Amorphastilbol was not previously known to be bioactive but does exhibit chromatographic behaviour and a positive modified Duquenois colour test such that possible forensic confusion with *Cannabis sativa* could occur [3]. The structure of amorphastilbol had been assigned from spectroscopic considerations. While there seemed little doubt that the assignment was correct, we have now confirmed it by a chemical interconversion with the known bibenzyl (**5**) from *Radula variabilis* [4] by methylation (to **2**) and chemical reduction to the dimethyl ether of **5** (**3**) using sodium amalgam. Likewise, the diacetyl ester of amorphastilbol (**4**) was similarly reduced and hydrolysed to produce the natural product itself (**5**). Identity was established by comparison of spectroscopic properties of the synthetic products with those published [4].

A fraction eluting from a silica gel column soon after amorphastilbol contained a biologically inactive substance which was different from the *A. fruticosa* constituents. This compound was shown to be 3,5-dihydroxy-7,4′-dimethoxyflavone (**6**). When **6** was methylated by ethereal diazomethane, 5-hydroxy-3,7,4′-trimethoxyflavone (**7**) was produced. The identical compound was produced by methylation of kaempferol (**8**; 3,5,7,4′-tetrahydroxyflavone). While **6** has been prepared a number of times by synthesis [5] and has been found in numerous other natural sources [6], it has not been reported previously as a constituent of *A. nana*. Some geographic differences between *A. nana* specimens was detected in that samples collected in eastern North Dakota were consistently more active than those from the central part of the state.

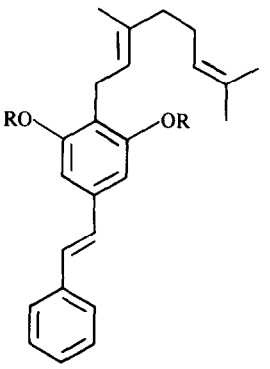
In vitro (agar dilution-streak) antimicrobial evaluation against a range of seven indicator micro-organisms demonstrated (Table 1) that amorphastilbol (**1**) possesses moderately potent activity against *Staphylococcus aureus* (Gram positive) and *Mycobacterium smegmatis* (acid fast). Dihydroamorphastilbol (**5**) is rather more bioactive. Compounds **6–8**, however, are inactive.

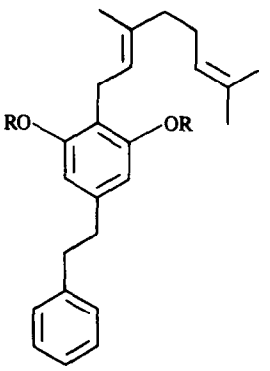
It is of some interest to note that the antimicrobially active constituents of *A. nana* and *A. fruticosa* contain no common component.

EXPERIMENTAL

Amorpha nana (Nutt.) was collected in Cass County, North Dakota, on 1 August 1983 by Dr. William T. Barker of North Dakota State University (Fargo, ND) and a specimen is on deposit at the Kansas Biological Survey Herbarium in Lawrence, KS.

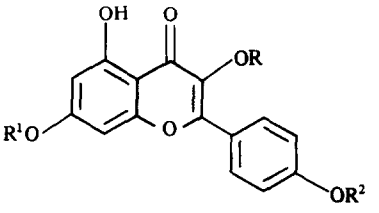
Isolation of amorphastilbol (**1**). Dried *A. nana* leaves (1020 g) were percolated to exhaustion with 95% EtOH. Evaporation of the biologically active percolate gave 126.5 g of a dark green viscous liquid. This (10 g) was partitioned between 5% HCl soln and CH_2Cl_2. The CH_2Cl_2 layer was dried (Na_2SO_4), filtered and evaporated under red. pres. to give *ca* 5 g of a dark green viscous liquid. This was partitioned between 90% MeOH and *n*-hexane to give 2.52 g of polar materials and 1.32 g of *n*-hexane soluble non-polar materials. The very viscous polar materials were dissolved in CH_2Cl_2–*n*-hexane (1:1) and chromatographed using silica gel G (100 g, 13 × 5 cm) and *n*-hexane–CH_2Cl_2 (4:1) with 7 ml fractions being collected. Fractions 5–7 contained a single bioactive substance Repeated recrystallization yielded 210 mg amorphastilbol (**1**) [1], mp 94–95° (reported [1], mp 94.5–95°); IR ν_{max}^{KBr} cm^{-1}: 3386 (OH), 1592 (Ar), 1449 (Ar); ^1H NMR (90 MHz, $CDCl_3$): δ1.59, 1.68, 1.81 (9H, *s*, CCH_3), 2.07 (2H, *s*, CH_2), 2.11 (2H, *s*, CH_2), 3.43 (2H, *d*, J = 7 Hz, CH_2), 5.09 (2H, *s*, exch. OH), 5.28 (2H, *t*, J = 7 Hz, C=CH), 6.57 (2H, *s*, ArH), 6.96 (2H, *s*, CH=CH–O) and 7.40 (5H, *m*,





1 R = H
2 R = Me
4 R = Ac

3 R = Me
5 R = H



6 R = H, $R^1 = R^2 = Me$
7 $R = R^1 = R^2 = Me$
8 $R = R^1 = R^2 = H$

Table 1. Antimicrobial activity of various *Amorpha nana* samples

	Micro-organisms* (μg/ml)						
Sample	1	2	3	4	5	6	7
A. nana, crude extract	1000	i	i	i	1000	i	i
A. nana, polar lipids	25	i	i	i	25	i	i
Amorphastilbol (**1**)	3.12	i	i	i	3.12	i	i
Amorphastilbol, dimethyl ether (**2**)	i	i	i	i	i	i	i
Amorphastilbol, diacetyl ester (**4**)	i	i	i	i	i	i	i
Dihydroamorphastilbol (**5**)	1.56	i	i	i	1 56	i	i
Dihydroamorphastilbol, dimethyl ether (**3**)	i	i	i	i	i	i	i
3,5-Dihydroxy-7,4′-dimethoxy flavone (**6**)	i	i	i	i	i	i	i
5-Hydroxy-3,7,4′-trimethoxyflavone (**7**)	i	i	i	i	i	i	i
Kaempferol (**8**)	i	i	i	i	i	i	i

*Micro-organism 1, *Staphylococcus aureus* ATCC 13709; 2, *Escherichia coli* ATCC 9637, *Salmonella gallinarum* ATCC 9184, *Klebsiella pneumoniae* ATCC 10031, *Mycobacterium smegmatis* ATCC 607, *Candida albicans* ATCC 10231 and *Pseudomonas aeruginosa* ATCC 27853. Crude extracts were tested by agar-dilution streak methods at 1000 and 100 μg/ml. Pure compounds were tested in the same manner starting at 100 μg/ml and diluting by a factor of two until an end-point was reached. The numbers refer to the last concentrations at which no visible growth occurred. Those substances or preparations which did not inhibit at the highest level tested are listed as inactive (i).

ArH); EIMS (probe) 70 eV, *m/z* (re. int.): 348 $[M]^+$ (15), 333 $[M-CH_3]^+$ (100), 305 (2), 291 (2), 279 $[CH_2CHC(CH_3)_2]^+$ (11), 265 $[M-CH_2CH_2CHC(CH_3)_2]^+$ (9), 263 (25), 251 (1), 237 (1), 225 $[M-CHCCH_3CH_2CH_2CH{=}C(CH_3)_2]^+$ (100). [Found: C, 82.83; H, 8 39. Calc for $C_{24}H_{28}O_2$ (348.46): C, 82.72; H, 8 10%.]

Methylation of amorphastilbol (**1**) Compound **1** (180 mg), Me_2SO_4 (80 mg), Me_2CO (15 ml) and K_2CO_3 (1 g) were refluxed for 6 hr, filtered, the filtrate washed with Me_2CO, and the filtrate and washings evaporated under red. pres The residue was dissolved in Et_2O (20 ml), washed well with H_2O (10 × 3 ml), dried, the solvent removed and the residue crystallized from *n*-hexane to give white needles of amorphastilbol dimethyl ether (**2**) (175 mg), mp 65–66.5°; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3019, 2961, 2930, 2872, 2857, 1578, 1456, 1417, 1225, 1217, 1169, 789, 731, 695, 671, ^1H NMR (90 MHz, $CDCl_3$): δ1.57, 1.64, 1.76 (9H, *s*, CH_3), 1.98 (4H, *br s*, CH_2CH_2), 3 34 (2H, *d*, *J* = 7 Hz, $ArCH_2$), 3 85 (6H, *s*, 2 × OCH_3), 5.20 (2H, *br t*, *J* = 7 Hz, CH), 6 69 (2H, *s*, ArCH=CHAr'), 7.04 (2H, *s*, ArH), 7.21–7.49 (5H, *m*, ArH); EIMS (probe) 70 eV, *m/z* (rel. int.): 376 $[M]^+$ (20), 307 $[M-C_5H_9]^+$ (26), 253 $[M-C_9H_{15}]^+$ (80), 69 $[C_5H_9]^+$ (51).

Selective reduction of amorphastilbol dimethyl ether (**2**) *to the corresponding bibenzyl* (**3**). Compound **2** (120 mg) was dissolved in EtOH (20 ml), 3.5% Na amalgam (600 mg) added and the reaction mixture stirred for 4 hr at 60°, cooled and then filtered. The residue was washed with EtOH (2 × 10 ml) and the filtrate and washings were evaporated. The residue was dissolved in $CHCl_3$ (40 ml), washed with H_2O, dried and evaporated to yield the viscous oily bibenzyl (120 mg); IR ν_{max}^{neat} cm^{-1}: 2950, 2900, 1620, 1595, 1500, 1460, 1425, 1380, 1350, 1320, 1225, 1190, 1165, 1115, 1075, 1025, 970, 880, 810, 740; ^1H NMR (90 MHz, $CDCl_3$): δ1 57, 1 64, 1.75 (9H, *s*, CCH_3), 1 97 (4H, *br s*, CH_2CH_2), 2.89 (4H, *s*, $ArCH_2CH_2$), 3 31 (2H, *d*, *J* = 7 Hz, CH_2CH), 3.76 (6H, *s*, 2 × OCH_3), 5.20 (2H, *br t*, *J* = 7 Hz, CH=CH_2), 6 34 (2H, *s*, ArH), 7.23 (5H, *s*, ArH); EIMS (probe) 70 eV, *m/z* (rel int): 378 $[M]^+$ (1), 309 $[M-C_5H_9]^+$ (5), 295 $[M-C_6H_{11}]^+$ (2), 255 $[M-C_9H_{15}]^+$ (73), 105 (100), 91 (100), 69 (57)

Acetylation of amorphastilbol. Amorphastilbol (0.5 g), pyridine (3.5 ml) and Ac_2O (3 5 ml) were stirred overnight at room temp The reaction mixture was poured over crushed ice and extracted with Et_2O (15 ml) The Et_2O was washed well with H_2O, dried and evaporated to give diacetylamorphastilbol as a viscous liquid (0.5 g) IR ν_{max}^{film} cm^{-1}: 3050, 2940, 2880, 1770, 1615, 1560, 1490, 1445, 1415, 1360, 1285, 1250, 1200, 1150 (sh), 1110, 1070 (sh), 1025, 945, 875, 730; ^1H NMR (90 MHz, $CDCl_3$): δ1 58, 1.66, 1 70 (9H, *s*, CCH_3), 1.98 (4H, *br s*, CH_2CH_2), 2.28 (6H, *s*, 2 × $COCH_3$), 3.18 (2H, *d*, *J* = 7 Hz, CH_2CH=), 5.00 (2H, *br t*, *J* = 7 Hz, CH=CH_2), 7.00 (2H, *s*, ArH), 7 09 (2H, *s*, CH=CHPh), 7.10–7.52 (5H, *m*, ArH); EIMS (probe) 70 eV, *m/z* (rel int.): 432 $[M]^+$ (4), 390 (2), 389 (2), 347 (2), 309 (10), 267 (20), 225 (40), 123 (30), 69 (55).

Selective reduction of diacetylamorphastilbol to bibenzyl. Diacetylamorphastilbol (250 mg), 3.5% Na amalgam (1 g) and MeOH (10 ml) were stirred for 4 hr at 55–60°, cooled and filtered. The filtrate was washed with MeOH (5 × 3 ml) and the filtrate and washings evaporated The residue was dissolved in Et_2O (15 ml), washed with H_2O, dried and evaporated to give the crude viscous liquid bibenzyl (180 mg). Pure colourless liquid bibenzyl (168 mg) was obtained by flash chromatography on silica gel (30 g) using CH_2Cl_2–hexane (1:2); IR ν_{max}^{film} cm^{-1}: 3500, 2990, 2970, 2900, 1640, 1600, 1530, 1505, 1455, 1390, 1350, 1265, 1225, 1170, 1035, 990 (sh), 905, 820; ^1H NMR (90 MHz, $CDCl_3$): δ1.67, 1.79, 1.80 (9H, *s*, CCH_3), 2.06 (4H, *s*, CH_2CH_2), 2.81 (4H, *br s*, CH_2CH_2), 3.39 (2H, *d*, *J* = 7 Hz, CH_2C=), 5.06 (2H, *s*, exch. OH), 5.25 (2H, *br t*, *J* = 7 Hz, CH=CH_2), 6.24 (2H, *s*, ArH), 7.00–7.50 (5H, *br s*, ArH); EIMS (probe) 70 eV, *m/z* (rel. int.): 350 $[M]^+$ (6), 281 (2), 265 (12), 227 (52), 123 (22), 105 (40), 91 $[C_6H_5CH_2]^+$ (90), 69 (52), 41 (100).

Isolation of 3,5-*dihydroxy-7,4'-dimethoxyflavone* (**6**). Fractions 9–13 also contained a single material so were combined, evaporated and repeatedly recrystallized to give 30 mg antimicrobially inactive 3,5-dihydroxy-7,4'-dimethoxyflavone (**6**). After serial recrystallization from CH_2Cl_2–*n*-hexane, **6** had mp 178–179°; UV λ_{max}^{MeOH} nm (log ε): sh 254 (4 35), 266 (4.43), sh 320 (4.21), 363 (4.47); $\lambda_{max}^{1\%\,NaOH}$ nm (log ε): sh 250 (4.50), sh 324 (3.86), 404 (4.41); $\lambda_{max}^{AlCl_3}$ nm (log ε): 270 (4.49), sh 304 (3.97), 350 (4.18) and 420 (4.53); IR ν_{max}^{KBr} cm^{-1}: 3312, 1649, 1620, 1500, etc; ^1H NMR (90 MHz, $CDCl_3$): δ3.88 (6H, *s*, OCH_3), 6.37 (1H, *d*, *J* = 2 Hz, ArH), 6.48 (1H, *d*, *J* = 2 Hz, ArH), 6.57 (1H, *s*, OH), 7.01 (2H, *d*, *J* = 7 Hz, ArH), 8.16 (2H, *d*, *J* = 9 Hz, ArH), 11.71 (1H, *s*, exch. OH); ^{13}C NMR (DMSO-d_6): δ55.27, 55.87, 91.93, 97.32, 104.03, 113.91, 123 11, 123.16, 129.26, 136.27, 156.09, 160.39, 160.50, 160.56, 164 89, 164.93, 176.03; EIMS (probe) 70 eV, *m/z* (rel. int.): 314 $[M]^+$ (100). [Found: C, 64.87; H, 4 51. Calc. for $C_{17}H_{14}O_6$: C, 64.97, H, 4.49%.]

Methylation of kaempferol (**8**). A sample (92.0 mg) of 3,5,7,4'-tetrahydroxyflavone (kaempferol, purchased from ICN Pharmaceuticals), was suspended in 10 ml Et_2O and treated with CH_2N_2 in Et_2O at 0° for 3 hr. After evaporation, the residue was dissolved in CH_2Cl_2 and chromatographed on silica gel G (5.5 g) using *n*-hexane–EtOAc (3:1) to yield 77.3 mg methylated product. After repeating the chromatographic step, the product was crystallized from *n*-hexane–CH_2Cl_2 to give 28.5 mg 5-hydroxy-3,7,4'-trimethoxyflavone (**7**): mp 144.5–146.5°; IR ν_{max}^{KBr} cm^{-1}: 3449, 3081, 3002, 2982, 2944, 2845, 1659, 1601, 1588, 1498, 1456, 1429, 1381, 1345, 1318, 1306, 1260, 1219, 1206, 1179, 1142, 1119, 1094, 1030, 1001, 943, 878, 834, 831, 789, 642; ^1H NMR (90 MHz, $CDCl_3$): δ3.86 (9H, *s*, OCH_3), 6.34 (1H, *d*, *J* = 2 Hz, ArH), 6 43 (1H, *d*, *J* = 2 Hz, ArH), 7.01 (3H, *d*, *J* = 9 Hz, ArH), 8.06 (2H, *d*, *J* = 9 Hz, ArH), 12.63 (1H, *s*, OH). [Found: C, 65.75; H, 4.94. Calc. for $C_{18}H_{16}O_6$ (328.31): C, 65.85; H, 4.91%.]

Methylation of 3,5-*dihydroxy-7,4'-dimethoxyflavone* (**6**). A sample of 7,4'-dimethoxy-3,5-dihydroxyflavone (100 mg, isolated as detailed above) was treated with ethereal CH_2N_2 as described above. The chromatographed product was crystallized from CH_2Cl_2–*n*-hexane to give 55.4 mg pure **7**, mp 144.5–146.5° alone or admixed with the product prepared by methylation of kaempferol. Likewise, the IR, ^1H NMR, MS and microanalysis were identical to that of synthetically prepared 5-hydroxy-3,7,4'-trimethoxyflavone (**7**).

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REFERENCES

1. Mitscher, L. A., Park, Y. H., Al-Shamma, A., Hudson, P. B. and Haas, T (1981) *Phytochemistry* **20**, 781.
2. Mitscher, L. A., Al-Shamma, A., Haas, T., Hudson, P. B. and Park, Y. H. (1979) *Heterocycles* **12**, 1033.
3. Kemal, M., Wahba Khalil, S. K., Rao, N. G. S. and Woolsey, N. F. (1979) *J. Nat. Prod.* **42**, 463.
4. Asakawa, Y., Toyota, M. and Takemoto, T. (1978) *Phytochemistry* **17**, 2005.
5. Dave, K. G., Telang, S. A. and Venkataramanam, K. (1960) *J. Sci. Ind. Res. (India)* **19b**, 470.
6. Nair, A. G. R., Ramesh, P., Subramanian, S. S. and Joshi, B. S. (1978) *Phytochemistry* **17**, 591.