# Activity-Guided Isolation of Steroidal Alkaloid Antiestrogen-Binding Site Inhibitors from *Pachysandra procumbens*

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Four novel steroidal alkaloids, (+)−(20*S*)-20-(dimethylamino)-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one (**1**), (+)−(20*S*)-20-(dimethylamino)-16α-hydroxy-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one (**2**), (+)−(20*S*)-3-(benzoylamino)-20-(dimethylamino)-5α-pregn-2-en-4*β*-yl acetate (**3**), and (+)−(20*S*)-2α-hydroxy-20-(dimethylamino)-3*β*-phthalimido-5α-pregnan-4*β*-yl acetate (**4**), as well as five known compounds, (−)-pachyaximine A (**5**), (+)-spiropachysine (**6**), (+)-axillaridine A (**7**), (+)-epipachysamine D (**8**), and (+)-pachysamine B (**9**), were isolated from *Pachysandra procumbens*, using a bioassay-guided fractionation based on inhibition of $^3$H-tamoxifen binding at the antiestrogen binding site (AEBS). Compounds **1**−**7** and **9** demonstrated significant activity as AEBS-inhibitory agents, and compounds **3**, **5** and **9** were found to potentiate significantly the antiestrogenic effect mediated by tamoxifen in cultured Ishikawa cells. The structure elucidation of compounds **1**−**4** was carried out by spectral data interpretation.

Tamoxifen is currently the most widely used antiestrogenic agent for the therapy of hormone receptor-positive advanced breast cancer and as an adjuvant treatment in postmenopausal breast-cancer patients.[1] In addition, in a recent study conducted with more than 13 000 women at risk for developing breast cancer, it was concluded that tamoxifen can reduce the incidence of this disease by approximately 45%.[2] The mechanism of action has not been thoroughly delineated, but major effects include both competition with endogenous estradiol by binding to the estrogen receptor[3] and blockage of breast-cancer cells in the $G_1$-phase of the cell cycle.[4,5] Apart from binding to the estrogen receptors, triphenylethylene antiestrogens have been shown to interact with high affinity and specificity to the microsomal antiestrogen binding site (AEBS).[6,7] AEBS receptors are found in a wide range of human tissues[8] and may play an important role in the mechanism of acquiring resistance to antiestrogen therapy.[9]

Cancer chemoprevention can be defined as the prevention, delay, or reversal of cancer by ingestion of dietary or pharmaceutical agents capable of modulating the process of carcinogenesis.[10,11] As part of our current work in this area, a battery of mechanism-based in vitro assays is employed to facilitate the discovery of potential cancer chemoprevention agents.[11] As exemplified by tamoxifen, one method of preventing tumor progression associated with breast cancer is to exploit the hormone-dependence of this disease.[11] Accordingly, we have employed the Ishikawa cell line (an endometrial adenocarcinoma cell line) to search for new antiestrogens.[12] An ancillary approach involves the use of compounds that bind specifically to the AEBS,[9] because, in principle, combining such agents with tamoxifen could elevate the concentration of this drug available for binding to the estrogen receptor.[9] In this regard, we have examined the potential of test agents to interact with the AEBS.

*Pachysandra* is a small genus of four species of shrubs and perennial herbs. Three species are indigenous to eastern Asia, occurring in mainland China, Japan, and Taiwan (*P. terminalis* Sieb. et Zucc., *P. axillaris* Franch., and *P. stylosa* Dunn.).[13] *P. procumbens* Michx. (Buxaceae), a native American ornamental, is a clump-forming adaptable ground-cover plant, valued for its bluish or grayish green foliage.[14] There have been no previous phytochemical or biological studies on this species. Several steroidal (3,20*S*)-diamino-5α-pregnane alkaloids, however, have been isolated previously from two related Asian species, *P. terminalis* Sieb. et Zucc.[15−19] and *P. axillaris* Franch.[20−23]

In the current investigation, the entire plant of *P. procumbens* was chosen for activity-guided fractionation. The petroleum ether- and ethyl acetate-soluble extracts were found to exhibit significant activity in an AEBS assay, as measured by inhibition of $^3$H-tamoxifen binding.[24] Four novel steroidal alkaloids, namely, (+)−(20*S*)-20-(dimethylamino)-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one (**1**), (+)−(20*S*)-20-(dimethylamino)-16α-hydroxy-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one (**2**), (+)−(20*S*)-3-(benzoylamino)-20-(dimethylamino)-5α-pregn-2-en-4*β*-yl acetate (**3**), and (+)−(20*S*)-2α-hydroxy-20-(dimethylamino)-3*β*-phthalimido-5α-pregnan-4*β*-yl acetate (**4**), have been isolated and characterized structurally. Five known compounds, (−)-pachyaximine A (**5**), (+)-spiropachysine (**6**), (+)-axillaridine A (**7**), (+)-epipachysamine D (**8**), and (+)-pachysamine B (**9**), were also isolated. The structure elucidation of **1**−**4** and the biological evaluation of **1**−**9** are reported in this communication.

## Results and Discussion

A molecular formula of $C_{29}H_{46}N_2O_2$ was determined from the HREIMS data (*m*/*z* 454.3568) for compound **1**. Comparison of its UV, IR, and $^1$H NMR data with pachystermine A[17] and several more recently isolated analogues indicated that **1** was a steroidal alkaloid.[20,21,25,26] The functional groups present in the molecule of **1** could be assigned as an amide carbonyl (IR, $\nu_{max}$ 1635 cm$^{-1}$; $\delta_C$ 169.5), a conjugated ketone ($\delta_C$ 196.7), a four-membered nonfused *β*-lactam ring (IR, $\nu_{max}$ 1732 cm$^{-1}$), and a *gem*-dimethyl group ($\nu_{max}$ 1336−1231 cm$^{-1}$; $\delta_C$ 20.1, 19.9; $\delta_H$ 1.06, 0.96). The $^1$H and $^{13}$C NMR data for the *β*-lactam moiety [$\delta_C$ 169.5, $\delta_C$ 57.8; $\delta_H$ 3.00, $\delta_C$ 46.5; $\delta_H$ 3.40 and 4.00]

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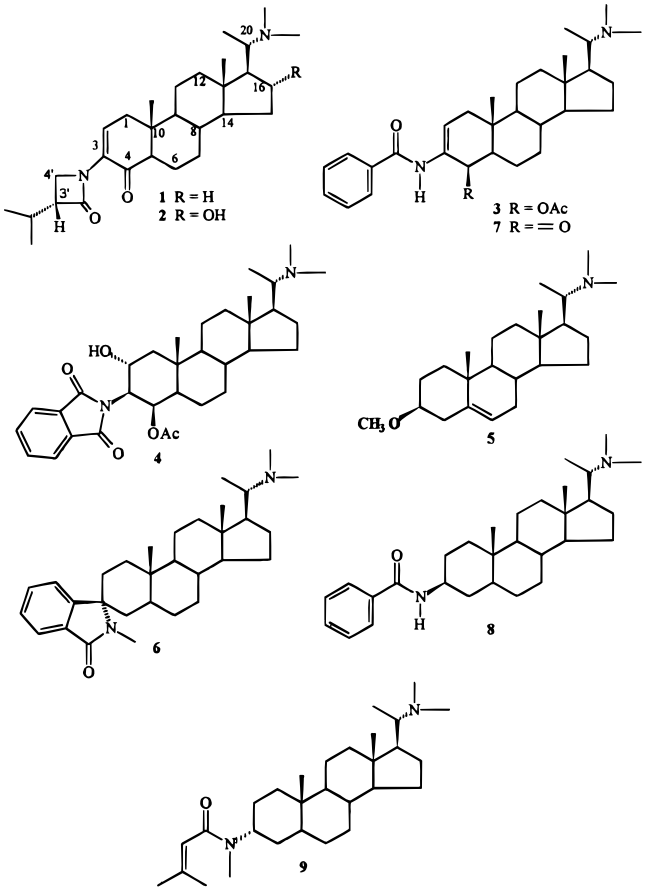


were consistent with literature values.[27] In the $^1$H NMR spectrum of **1**, two tertiary methyl signals ($\delta$ 0.65 and 0.88) and a secondary methyl signal [$\delta$ 0.87 ($J$ = 6.3 Hz)] corresponded to Me-18, Me-19, and Me-21, respectively. A 6H singlet was assigned to two *N*-methyl groups ($\delta_H$ 2.17; $\delta_C$ 39.9) by data comparison with pachyaximine A (**5**).[22,25] The determination of the $^{13}$C NMR assignments for the ring-A carbons, and the establishment of the linkage of the $\beta$-lactam moiety at C-3 were carried out by comparison with NMR data published for axillaridine A (**7**).[21] The C-2 olefinic proton of **1** appeared at $\delta_H$ 7.28 as a doublet of doublets ($J$ = 2.5, 6.8 Hz), supporting the fact that the carbonyl group of the $\alpha,\beta$-unsaturated carbonyl moiety was at C-4 and not at C-1.[21] In a $^1$H−$^{13}$C HMQC NMR experiment, a doublet of doublets at $\delta_H$ 7.28 (H-2) correlated to the carbon peak at $\delta_C$ 129.8, whereas $\delta_H$ 3.00 (H-3′) correlated to $\delta_C$ 57.8. In addition, the signals at $\delta_H$ 3.40 and 4.00 (H-4′) correlated to $\delta_C$ 46.5, and the methyl group resonances at $\delta_H$ 1.06 and 0.96 showed cross peaks with signals at $\delta_C$ 20.1 and 19.9, respectively. HMBC experiments allowed the complete assignments of the $^1$H and $^{13}$C NMR spectra of **1**, and a summary of all HMBC correlations observed for **1** is also shown in Table 1. The stereochemistry of the same type of lactam ring as found in **1** was confirmed for the model compound pachystermine A by chemical degradation and partial synthesis.[17] Thus, the structure of compound **1** was assigned as (+)−(20*S*)-20-(dimethylamino)-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one.

An elemental formula of $C_{29}H_{46}N_2O_3$ for **2** was consistent with the HREIMS data ([M$^+$] $m/z$ 470.3503) obtained. Comparison of the IR and $^1$H and $^{13}$C NMR data of **2** with those of compound **1** suggested they were closely related steroidal alkaloids, with the only difference being the presence of an additional hydroxyl group (IR, $\nu_{max}$ 3442 br cm$^{-1}$) in **2**. The relative location of the OH proton of **2** was confirmed by an HMBC experiment, in which correlations were observed for the resonance at $\delta_H$ 4.30 (H-16) with the signals of $\delta_C$ 58.3 (C-17) and 41.6 (C-13). In turn, the H-17 signal at $\delta_H$ 1.25 correlated with the signals at $\delta_C$ 71.6 (C-16), 41.6 (C-13), 57.4 (C-20), 53.9 (C-14), and 34.9 (C-15). In a homonuclear decoupling experiment, irradiation at $\delta_H$ 4.30 (H-16) resulted in the collapse of H-17 (multiplet) into a doublet ($J_{17,20}$ = 11.2 Hz). In addition, H-16 ($\delta_H$ 4.30) showed a cross peak with H-17 ($\delta_H$ 1.25 m) in a DQCOSY experiment, again suggesting the location of the OH at C-16. The presence of a C-16 OH group was confirmed for **2** by comparison with published $^{13}$C NMR data for the model compound spiropachysine B at the C-14 through C-17 and C-20 positions.[28] The stereochemistry of the C-16 hydroxyl in **2** was investigated by a 1D NOE NMR study, as well as molecular modeling experiments on both the α and $\beta$ epimers at C-16, in which the vicinal $^1$H NMR coupling constants for H-15α, H-15$\beta$, H-16, and H-17α were calculated using the Altona equation.[29] In the 1D NOE experiment, irradiation at H-16 ($\delta_H$ 4.30) gave no enhancement with H-17α, suggesting that H-16 is $\beta$. From the molecular modeling study, the predicted values for the 16α-OH epimer were $J_{16\beta,17\alpha}$ = 4.7 Hz, $J_{16\beta,15\beta}$ = 7.0 Hz, and $J_{16\beta,15\alpha}$ = 1.2 Hz. These values were consistent with the doublet of doublets of doublets for H-16 ($J$ = 7.7, 6.1, 1.6 Hz) actually observed in the $^1$H NMR spectrum of **2**. In contrast, for the 16$\beta$-OH epimer, the calculated values were $J_{16\alpha,17\alpha}$ = 6.5 Hz, $J_{16\alpha,\ 15\alpha}$ = 9.7 Hz, and $J_{16\alpha,15\beta}$ = 5.1 Hz.[29] These calculations were made based on published values for a bisdesmosidic cholestane glycoside, which bears a 16$\beta$-hydroxyl functionality (H-16; $\delta_H$ 4.30; $J$ = 7.9, 7.9, 4.9 Hz).[30] Thus, the structure of compound **2** was assigned as (+)−(20*S*)-20-(dimethylamino)-16α-hydroxy-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one.

A molecular formula of $C_{32}H_{46}N_2O_3$ was determined by HREIMS ($m/z$ 506.3508) for compound **3**. Comparison of its UV, IR, and $^1$H and $^{13}$C NMR data indicated that it was a steroidal alkaloid closely related in structure to epipachysandrine A.[15,26] Furthermore, it was apparent that in the molecule of **3** were an NH group (IR, $\nu_{max}$ 3402 cm$^{-1}$; $\delta_H$ 8.40), an amide carbonyl ($\nu_{max}$ 1664 cm$^{-1}$; $\delta_C$ 166.0), and an acetate group (IR, $\nu_{max}$ 1712 cm$^{-1}$; $\delta_C$ 174.0, 21.0; $\delta_H$ 2.12). $^1$H and $^{13}$C NMR data for a benzoyl moiety in **3** were consistent with literature values.[21,26] The linkage of this benzoyl moiety to C-3 (ring A) in the molecule of **3** was suggested by comparison with axillaridine A (**7**).[21] The C-2 olefinic proton appeared at $\delta_H$ 7.00 as a double doublet ($J$ = 2.2, 6.0 Hz) supporting the fact that the acetate group was located at C-4 and not at C-1.[21] This observation was confirmed from HMBC and homonuclear decoupling NMR experiments. The HMBC experiments allowed the complete assignments of the $^1$H and $^{13}$C NMR spectra of **3** (Table 2). The H-4 signal at $\delta_H$ 5.18 exhibited cross peaks with signals at $\delta_C$ 115.0 (C-2) and $\delta_C$ 130.0 (C-3), and H-1 ($\delta_H$ 2.34) with $\delta_C$ 115.0 (C-2), $\delta_C$ 130.0 (C-3), and $\delta_C$ 45.5 (C-5). The NH signal ($\delta_H$ 8.40) showed a connectivity with $\delta_C$ 115.0 (C-2), consistent with the results of a homonuclear decoupling NMR experiment, where irradiation at H-2 ($\delta_H$ 7.00) resulted in H-1 [$\delta_H$ 2.34 (dd, $J$ = 6.0, 18.2 Hz)] collapsing to a doublet ($J$ = 18.2 Hz). In a 1D NOE NMR experiment, irradiation of H-4 ($\delta_H$ 5.18) did not lead to any enhancement, suggesting that H-4 was in the α position. Furthermore, the coupling constant for H-4 ($\delta_H$ 5.18, $J_{4\alpha,5}$ = 2.9 Hz) strongly supported this stereochemical assignment, because the coupling constant for H-$\beta$ was $J_{4\beta,5}$ = 10.5−12 Hz.[15,26] Thus, the structure of compound **3** was

**Table 1.** $^1$H and $^{13}$C NMR Data in $CDCl_3$ for Compounds **1** and **2**, and HMBC Correlations for **1**[a]

| carbon | **1** $\delta_H$ (mult.) *J* (Hz) | **1** $\delta_C$ | **2** $\delta_H$ (mult.) *J* (Hz) | **2** $\delta_C$ | carbon signal correlated[b] |
|---|---|---|---|---|---|
| 1 | 2.45 dd (5.9, 18.0) | 39.3 | 2.45 dd (5.9, 18.0) | 39.1 | C-2, C-3, C-5, C-10 |
| 2 | 7.28 dd (2.5, 6.8) | 129.8 | 7.28 dd (2.5, 6.8) | 130.5 | C-4, C-3, C-10 |
| 3 | | 131.7 | | 131.4 | |
| 4 | | 196.7 | | 196.7 | |
| 5 | | 55.8 | | 55.6 | |
| 6 | | 20.4 | | 20.2 | |
| 7 | | 30.5 | | 30.4 | |
| 8 | | 34.7 | | 34.0 | |
| 9 | | 54.1 | | 53.0 | |
| 10 | | 40.0 | | 40.0 | |
| 11 | | 20.8 | | 20.5 | |
| 12 | | 39.5 | | 39.7 | |
| 13 | | 41.6 | | 41.6 | |
| 14 | | 56.2 | | 53.9 | |
| 15 | | 23.9 | | 34.9 | |
| 16 | | 27.6 | 4.30 ddd (1.6, 6.1, 7.7) | 71.6 | |
| 17 | 1.40 m | 54.8 | 1.25 m | 58.3 | C-16, C-14, C-20, C-15, C-13 |
| 18 | 0.65 s | 13.3 | 0.87 s | 13.7 | |
| 19 | 0.88 s | 12.3 | 0.88 s | 13.2 | |
| 20 | 2.45 m | 61.1 | 3.05 m | 57.4 | C-17, C-13 |
| 21 | 0.87 d (6.3) | 9.9 | 1.01 d (6.3) | 10.1 | C-20, C-17 |
| *N*−$Me_2$ | 2.17 s | 39.9 | 2.36 s | 39.9 | |
| N−C=O | | 169.5 | | 169.5 | |
| Me-5′ | 1.06, 0.96 d (6.6) | 19.9, 20.1 | 1.06, 0.96 d (6.6) | 19.9, 20.1 | 20.1, 19.9, C-5′, C-3′ |
| 3′ | 3.00 m | 57.8 | 3.00 m | 57.6 | N−C=O |
| 4′ | 3.40 dd (2.8, 6.8); 4.00 dd (5.6, 6.8) | 46.5 | 3.40 dd (2.8, 6.8); 4.00 dd (5.6, 6.8) | 46.5 | N−C=O, C-3′ |
| 5′ | | 28.3 | | 28.3 | |

[a] TMS was used as the internal standard; chemical shifts are shown in the $\delta$ scale with *J* values in parentheses. [b] Data show carbon signals correlated to proton indicated.

**Table 2.** $^1$H and $^{13}$C NMR Data and HMBC Correlations in $CDCl_3$ for Compound **3**[a]

| carbon | $\delta_H$ (mult.) *J* (Hz) | $\delta_C$ | carbon signal correlated[b] |
|---|---|---|---|
| 1 | 2.34 dd (6.0, 18.2) | 39.1 | C-2, C-3, C-5 |
| 2 | 7.00 dd (2.2, 6.0) | 115.0 | C-4, C-3, C-10 |
| 3 | | 130.0 | |
| 4 | 5.18 d (2.9) | 72.6 | C-3, C-2 |
| 5 | | 45.5 | |
| 6 | | 24.0 | |
| 7 | | 31.7 | |
| 8 | | 31.9 | |
| 9 | | 54.4 | |
| 10 | | 33.8 | |
| 11 | | 20.8 | |
| 12 | | 39.5 | |
| 13 | | 41.6 | |
| 14 | | 56.4 | |
| 15 | | 24.0 | |
| 16 | | 27.6 | |
| 17 | 1.40 m | 54.7 | C-13, C-20 |
| 18 | 0.67 s | 12.2 | C-14, C-17, C-13, C-12 |
| 19 | 1.03 s | 13.8 | C-1, C-10, C-9 |
| 20 | 2.44 m | 61.1 | |
| 21 | 0.87 d (6.3) | 9.9 | C-20, C-17 |
| N−$Me_2$ | 2.17 s | 39.9 | C-20, 39.9 |
| N−C=O | | 166.0 | |
| OAc | 2.12 s | 21.0 | |
| OAc-4 | | 174.0 | |
| N−H | 8.40 s | | C-2 |
| 1′ | | 134.6 | |
| 2′ | 7.80 br d (7.3) | 128.7 | C-4′, C-3′, C=O |
| 3′ | 7.46 br dd (7.3, 7.3) | 126.9 | C-1′, C-2′, C-5′ |
| 4′ | 7.49 br dd (7.3, 7.3) | 131.5 | |
| 5′ | 7.46 br dd (7.3, 7.3) | 126.9 | C-1′, C-3′, C-6′ |
| 6′ | 7.80 br d (7.3) | 128.7 | C-4′, C-5′, C=O |

[a] TMS was used as the internal standard; chemical shifts are shown in the $\delta$ scale with *J* values in parentheses. [b] Data show carbon signals correlated to proton indicated.

assigned as (+)−(20*S*)-3-(benzoylamino)-20-(dimethylamino)-5α-pregn-2-en-4$\beta$-yl acetate.

Compound **4** was assigned a molecular formula of $C_{33}H_{46}N_2O_5$ from its HREIMS data ($[M]^+$ *m/z* 550.3406). In its $^1$H NMR spectrum, the presence of two tertiary methyl groups ($\delta_H$ 0.69 and 1.19) and a secondary methyl group ($\delta_H$ 0.89) corresponded to Me-18, Me-19, and Me-21, respectively. An acetate group ($\delta_C$ 170.8, 20.8; $\delta_H$ 1.98) and an amide carbonyl ($\delta_C$ 168.8) functionality were also present. The relative locations of these functionalities were established from the following observations. In an HMQC experiment, the signal at $\delta_H$ 5.76 correlated with that at $\delta_C$ 74.2 (C-4), and other correlations were $\delta_H$ 4.34 with $\delta_C$ 58.9 (C-3), and $\delta_H$ 4.58 with $\delta_C$ 64.4 (C-2). $^1$H and $^{13}$C NMR data for the phthalimide moiety in the molecule of **4** were consistent with data for the model compound, phthalic anhydride.[31] The resonances at $\delta_C$ 168.8 (N−C=O), 132.2 (C-1′ and C-6′), 134.4 (C-3′ and C-4′), and 123.7 (C-2′ and C-5′) appeared as double intensity peaks, along with aromatic protons at $\delta_H$ 7.82 (H-2′ and H-5′) and $\delta_H$ 7.69 (H-3′ and H-4′), supporting the presence of a phthalimide group.[31] HMBC experiments allowed the complete assignments of the location of the phthalimide group in **4** (Table 3). The stereochemistry at H-4 was confirmed from a ROESY NMR experiment, in which H-4 ($\delta_H$ 5.76) showed a cross peak with H-5α ($\delta_H$ 1.92), suggesting that H-4 is in the α orientation, with a $^1$H NMR coupling constant of $J_{3,4}$ = 5.6 Hz.[26] In addition, in a series of homonuclear decoupling experiments, irradiation at $\delta_H$ 4.34 (H-3) resulted in the collapse of $\delta_H$ 5.76 from a doublet of doublets ($J$ = 5.6, 6.8 Hz) to a doublet ($J$ = 5.6 Hz). The α configuration of H-3 ($\delta_H$ 4.34) was supported by the $^1$H NMR coupling constant of $J_{3,4}$ = 6.8 Hz, and $J_{3,2}$ = 11.8 Hz. Moreover, irradiation at $\delta_H$ 5.76 (H-4) resulted in the doublet of doublets at $\delta_H$ 4.34 collapsing into a single doublet ($J$ = 11.8 Hz) and suggested that both H-2 and H-3 are axially oriented, with H-2 being $\beta$.[31] Thus, compound **4** was assigned as (+)−(20*S*)-2α-hydroxy-20-(dimethylamino)-3$\beta$-phthalimido-5α-pregnan-4$\beta$-yl acetate.

**Table 3.** $^1$H and $^{13}$C NMR Data and HMBC Correlations in $CDCl_3$ for Compound **4**[a]

| carbon | $\delta_H$ (mult.) $J$ (Hz) | $\delta_C$ | carbon signal correlated[b] |
|---|---|---|---|
| 1 | | 46.3 | |
| 2 | 4.58 m | 64.4 | |
| 3 | 4.34 dd (11.8, 6.8) | 58.9 | N−C═O, C-4 |
| 4 | 5.76 dd (5.6, 6.8) | 74.2 | OAc-4, C-3, C-10 |
| 5 | 1.92 m | 43.9 | |
| 6 | | 22.8 | |
| 7 | | 31.2 | |
| 8 | | 35.3 | |
| 9 | | 55.9 | |
| 10 | | 36.0 | |
| 11 | | 21.2 | |
| 12 | | 39.7 | |
| 13 | | 41.8 | |
| 14 | | 56.3 | |
| 15 | | 23.9 | |
| 16 | | 27.6 | |
| 17 | 1.40 m | 54.7 | |
| 18 | 0.69 s | 12.3 | C-14, C-12, C-13 |
| 19 | 1.19 s | 19.8 | C-10, C-5 |
| 20 | 2.40 m | 61.1 | |
| 21 | 0.89 d (6.4) | 9.9 | C-20, C-17 |
| N−$Me_2$ | 2.17 s | 39.9 | C-20, 39.9 |
| N−C═O | | 168.8 | |
| OAc | 1.98 s | 20.8 | |
| OAc-4 | | 170.8 | |
| 1′ | | 132.2 | |
| 2′ | 7.82 m | 123.7 | N−C═O, C-3′ |
| 3′ | 7.69 m | 134.4 | C-1′, C-2′, C-5′ |
| 4′ | 7.69 m | 134.4 | C-3′, C-6′, C-2′ |
| 5′ | 7.82 m | 123.7 | N−C═O, C-4′ |
| 6′ | | 132.2 | |

[a] TMS was used as the internal standard; chemical shifts are shown in the $\delta$ scale with $J$ values in parentheses. [b] Data show carbon signals correlated to proton indicated.

**Table 4.** Antiestrogen Binding Site Interaction and Modulation of Tamoxifen Antiestrogenic Activity in Ishikawa Cells by Compounds **1**−**9**

| compound | AEBS inhibitory activity[a] | potentiation of antiestrogenic activity mediated by tamoxifen in Ishikawa cells[b] |
|---|---|---|
| **1** | 2.8 | 0.11 |
| **2** | 3.5 | 0.11 |
| **3** | 0.6 | 0.035 |
| **4** | 6.8 | nd[c] |
| **5** | 0.4 | 0.07 |
| **6** | 7.4 | 0.19 |
| **7** | 8.0 | 0.16 |
| **8** | >20 | nd[c] |
| **9** | 0.6 | 0.037 |

[a] Concentration required to reduce the binding of [$^3$H]tamoxifen by 50% ($\mu$M). For additional details see the Experimental Section. [b] Concentration of tamoxifen ($\mu$M) required to inhibit estrogen-induced alkaline phosphatase activity by 50%. Assays were performed with various concentrations of tamoxifen (0.027−3.54 $\mu$M) in the presence of a fixed concentration of the indicated test compounds (compounds **3** and **9**, 1 $\mu$g/mL; all others 2 $\mu$g/mL). In the absence of test compounds, the $IC_{50}$ of tamoxifen itself was 0.42 $\mu$M. For additional details, see the Experimental Section.[c] nd, not determined.

The initial plant extracts showed negative results when evaluated with cultured Ishikawa (human endometrial adenocarcinoma) cells, indicating a lack of estrogenic or antiestrogenic activity. Compounds **1**−**7** and **9**, however, inhibited the binding of tamoxifen to the AEBS, with $IC_{50}$ values ranging from 0.4 to 8.0 $\mu$M (Table 4). Compounds **3**, **5**, and **9** were most potent in this capacity. Because a rapid increase of AEBS in breast tumors is one mechanism leading to tamoxifen resistance, compounds with the potential of selectively binding to the AEBS are of therapeutic interest.[9] To assess further the biological potential of compounds **1**−**9**, they were tested in the Ishikawa cell line (which has both AEBS and estrogen receptors) in combination with tamoxifen. The results demonstrated a good correlation with the AEBS data, with compounds **3**, **5**, and **9** greatly potentiating tamoxifen activity, as signified by a substantial decrease in the apparent $IC_{50}$ values of tamoxifen (Table 4). Active isolates are currently being evaluated in additional biological test systems to further characterize their potential clinical utility.

## Experimental Section

**General Experimental Procedures.** Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured on a Perkin−Elmer model 241 polarimeter. UV spectra were obtained with a Beckman DU-7 spectrometer. IR spectra were obtained with a Midac Collegian FT-IR spectrophotometer. $^1$H and $^{13}$C NMR spectra were measured with TMS as internal standard, using a Varian XL-300 instrument operating at 300 and 75.6 MHz, respectively. 1D NOE, $^1$H−$^1$H COSY, and $^1$H−$^{13}$C HETCOR NMR experiments were also performed on this instrument, using standard pulse sequences. $^1$H−$^{13}$C HMBC, HMQC, and ROESY NMR experiments were conducted on a Bruker Avance DPX-300 MHz spectrometer. EIMS and HREIMS were obtained on a Finnigan MAT 90 instrument.

**Plant Material.** Entire plants of *Pachysandra procumbens* were purchased from Holland American, Inc., Midlothian, VA, in October 1995. In addition, larger quantities of *P. procumbens*, obtained as seedlings from Greenwood Propagation, Hebron, IL, were generated by growing to maturity in a greenhouse. This plant was verified by Dr. D. D. Soejarto, College of Pharmacy, University of Illinois at Chicago. A voucher specimen (accession no. PC-0398) has been deposited at the University of Illinois Pharmacognosy Field Station.

**Assay for Antiestrogen-Binding Site Activity.** Frozen female Sprague−Dawley rat liver was homogenized in assay buffer [10 mM Tris, pH 7.4, 1 mM EDTA, 10% glycerol (v/v), 0.1% monothioglycerol (v/v)] and centrifuged 12 000 × $g$ for 30 min. The supernatant was diluted to a concentration equivalent to 1 g liver/40 mL buffer, and free estrogen receptors were blocked by incubating the preparation at 4 °C with $10^{-6}$ M estradiol for 1 h. Test samples were initially dissolved in DMSO (4 mg/mL), and diluted tenfold in assay buffer. Reaction mixtures were prepared containing test samples (20 $\mu$g/mL), [$^3$H]tamoxifen (0.04 $\mu$Ci; 83 Ci/mM), and 180 $\mu$L receptor preparation and were incubated overnight at 4 °C. Nonbound [$^3$H]tamoxifen was removed by adding an equal volume of 1% charcoal (w/v)/0.1% dextran (w/v) in buffer. After a 10-min incubation, the mixture was centrifuged, and the supernatant was removed and subjected to liquid scintillation counting. Data were analyzed by comparing specifically bound [$^3$H]tamoxifen of reaction mixtures containing test samples to solvent controls. Nonspecific binding was assessed by adding 20 $\mu$g/mL of unlabeled tamoxifen to the reaction mixture. If the test samples (20 $\mu$g/mL) inhibited binding by ≥50%, dose−response studies were performed to determine $IC_{50}$ values.[24] All assays were conducted in triplicate.

**Assay for Estrogen-Dependent Induction of Alkaline Phosphatase in Ishikawa Cells.** Ishikawa cells were routinely cultured in DMEM/F12 medium supplemented with 2 mM glutaMAX-1, antibiotic−antifungal (penicillin G sodium 10 units/mL, streptomycin sulfate 10 $\mu$g/mL, amphotericin B 0.25 $\mu$g/mL), 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS). All media components and reagents were purchased from Life Technologies Inc. (Gibco-BRL), Grand Island, NY, except FBS, which came from Atlanta Biologicals, Atlanta, GA. A day before plating the cells, the medium was changed to a phenol red-free formulation of DMEM/F12 medium containing charcoal/dextran stripped FBS to remove estrogens. Cell suspensions (190 $\mu$L containing 5 × $10^4$ cells) were plated into 96-well microtiter plates and allowed to settle

overnight. Test compounds (10 $\mu$L dissolved in DMSO, diluted tenfold in EtOH and then an additional tenfold in complete phenol red-free medium to reduce any inductive effects of DMSO and EtOH), $2 \times 10^6$ M estradiol, and the relevant controls were added to the plated cells and incubated for 4 days. The plates were processed by removing the test medium, washing twice with PBS, adding 50 $\mu$L of 0.1% Triton X-100 (v/v) in 0.1 M Tris buffer (pH 9.8), and freezing at −80 °C. For analysis, the plates were rapidly thawed to 37 °C, and 150 $\mu$L of 0.1 M Tris buffer (pH 9.8) containing 1 mg/mL of *p*-nitrophenyl phosphate was added to each well. The plates were monitored at 405 nm with an ELISA reader every 15 s with a 10 s shake between each reading for the first 8 min. The slopes of the obtained curves were calculated, and those obtained with cell preparations treated with test samples were compared to standards.[12] The percent induction was calculated as follows: % Induction = $(\text{Slope}_{\text{sample}} - \text{Slope}_{\text{cells}}/\text{Slope}_{\text{DMSO}} - \text{Slope}_{\text{cells}}) \times 100$. To evaluate potentiation of antiestrogenic activity, dose−response studies were performed with tamoxifen (0.027−3.54 $\mu$M), wherein each test concentration of tamoxifen was admixed with a fixed concentration of test compounds **1**−**9** (compounds **3** and **9**, 1 $\mu$g/mL; all others, 2 $\mu$g/mL). $IC_{50}$ values were calculated from dose−response curves.

**Extraction and Isolation.** The dried plant material (3.5 kg) was ground and extracted with MeOH−$H_2O$ (9:1; 3 × 9 L) by maceration. The resultant extracts were combined and concentrated to dryness in vacuo at 37 °C. The dried MeOH extract was extracted with petroleum ether to afford a dried petroleum ether-soluble residue (9.0 g). The MeOH extract was then suspended in $H_2O$ and partitioned with EtOAc to give, on drying, 80.0 g of an EtOAc-soluble residue. Both the petroleum ether- and EtOAc-soluble extracts demonstrated AEBS inhibitory activity, with $IC_{50}$ values of 0.8 $\mu$g/mL and 3.8 $\mu$g/mL, respectively. A second larger sample of the plant material (5.9 kg) was extracted according to the same procedure to afford dried petroleum ether (19.5 g), and EtOAc-soluble residues (132.0 g).

Fractionation of the petroleum ether extract (from the first batch of plant material, 8.5 g) was initiated by column chromatography over Si gel as stationary phase, using petroleum ether and EtOAc (0−100%) mixtures as eluents, with a final wash using 100% MeOH, to afford nine pooled fractions. The AEBS activity was found to be concentrated in fractions 8−9. Thus, fractions 8 and 9 were combined, and eluted with gradient mixtures of petroleum ether−EtOAc−triethylamine. From fraction 18, eluted with petroleum ether−EtOAc−triethylamine (98:2:1), (+)-axillaridine A was obtained (**7**, 18.5 mg, 0.00053%). Fraction 31 from the same column afforded (+)-epipachysamine D (**8**, 50.0 mg, 0.0014%), and was eluted with petroleum ether−EtOAc−triethylamine (95:4:1). Fraction 29 (semi-pure), eluted with petroleum ether−EtOAc−triethylamine (95:4:1), was subjected to passage over Sephadex LH-20 (elution with MeOH), resulting in the crystallization of compound **3** (8.5 mg, 0.00024%) from the mother liquor.

The EtOAc fraction (78.0 g) from the first plant sample was purified using Si gel as stationary phase and eluted with $CHCl_3$−MeOH−$NH_4OH$ (24%)mixtures of increasing polarity, to afford 10 pooled fractions. Fractions 3−5 were the most active subfractions in the AEBS assay. Fraction 6 from this first column afforded (−)-pachyaximine A (**5**, 19.0 mg, 0.00054%), by eluting with $CHCl_3$−MeOH−$NH_4OH$ (24%) (98:1:1). Fractions 3−5, eluted with $CHCl_3$−MeOH−$NH_4OH$ (24%) (95:4:1), were combined and chromatographed on a Si gel column developed with $CHCl_3$−MeOH−triethylamine (93:7:1) as solvent system, to afford, in turn, compound **2** (30.0 mg, 0.00085%) and (+)-spiropachysine (**6**, 12.0 mg, 0.00034%). Further purification of fraction 56, eluted from $CHCl_3$−MeOH−triethylamine (92:8:1), by passage over Sephadex LH-20 eluted with MeOH, resulted in the crystallization of compound **1** (32.5 mg, 0.00093%) from the mother liquor.

Fractionation of the petroleum ether extract from the second batch of plant material (19.5 g) was carried out by column chromatography over Si gel as stationary phase, using petroleum ether−EtOAc−triethylamine mixtures in a gradient with a final wash using 100% MeOH to afford 12 pooled fractions. Fractions 7−10 (82−90% inhibition) were combined, and eluted with petroleum ether−EtOAc−triethylamine mixtures in a gradient. Fraction 24 from the initial column, eluted with petroleum ether−EtOAc−triethylamine (98:2:1), was purified further by passage over Sephadex LH-20 eluted with MeOH, resulting in the crystallization of (+)-pachysamine B (**9**, 20.0 mg, 0.00034%) from the mother liquor. Fraction 61 from the same column afforded compound **4** (16.5 mg, 0.00028%), by eluting with petroleum ether−EtOAc−triethylamine (85:13:2).

**(+)-(20*S*)-20-(Dimethylamino)-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one (1):** colorless needles; mp 208 °C; $[\alpha]^{20}_D$ +41.7° (*c* 0.08, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 221 (3.96), 270 (3.75) nm; IR (film) $\nu_{max}$ 3420, 2984, 2916, 2849, 1732, 1635, 1558, 1435, 1336, 1231, 1089, 959 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) and $^{13}C$ NMR ($CDCl_3$, 75.4 MHz) data, see Table 1; EIMS (70 eV) *m/z* $[M]^+$ 454 (40), 439 (100), 425 (28), 367 (43), 297 (41), 148 (57), 136 (67), 134 (71); HREIMS *m/z* 454.3568 (calcd for $C_{29}H_{46}O_2N_2$, 454.3548).

**(+)-(20*S*)-20-(Dimethylamino)-16α-hydroxy-3-(3′α-isopropyl)-lactam-5α-pregn-2-en-4-one (2):** colorless needles; mp 205 °C; $[\alpha]^{20}_D$ +26.1° (*c* 0.07, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 220 (3.83), 275 (3.50) nm; IR (film) $\nu_{max}$ 3442 (br), 2954, 2898, 1745, 1688, 1480, 1384, 1328, 1040 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) and $^{13}C$ NMR ($CDCl_3$, 75.4 MHz) data, see Table 1; EIMS (70 eV) *m/z* $[M]^+$ 470 (9), 455 (100), 425 (69), 397 (23), 342 (51), 137 (32), 105 (44); HREIMS *m/z* 470.3503 (calcd for $C_{29}H_{46}O_3N_2$, 470.3497).

**(+)-20*S*-3-(Benzoylamino)-20-(dimethylamino)-5α-pregn-2-en-4β-yl acetate (3):** colorless needles; mp 179 °C; $[\alpha]^{20}_D$ +48.2° (*c* 0.04, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 224 (3.94), 264 (3.73) nm; IR (film) $\nu_{max}$ 3402, 2946, 2882, 2786, 1712, 1664, 1552, 1384, 1048 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) and $^{13}C$ NMR ($CDCl_3$, 75.4 MHz) data, see Table 2; EIMS (70 eV) *m/z* $[M]^+$ 506 (3), 491 (6), 359 (5), 159 (5), 105 (100); HREIMS *m/z* 506.3508 (calcd for $C_{32}H_{46}O_3N_2$, 506.3497).

**(+)-20*S*-2α-Hydroxy-20-(dimethylamino)-3β-phthalimido-5α-pregnan-4β-yl acetate (4):** colorless needles; mp 269 °C; $[\alpha]^{20}_D$ + 48.0° (*c* 0.03, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 228 (4.19), 243 (394), 293 (3.33) nm; IR (film) $\nu_{max}$ 3470, 2933, 2867, 2775, 1734, 1649, 1468, 1237, 1024, 955 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) and $^{13}C$ NMR ($CDCl_3$, 75.4 MHz) data, see Table 3; EIMS (70 eV) *m/z* $[M]^+$ 550 (13), 535 (54), 256 (32), 148 (100), 105 (60); HREIMS *m/z* 550.3406 (calcd for $C_{33}H_{46}O_5N_2$, 550.3395).

**(−)-Pachyaximine A (5):** colorless needles; mp 155 °C [lit.[25] 152 °C]; $[\alpha]^{20}_D$ −28.3° (*c* 0.06, $CHCl_3$) [lit.[25] $[\alpha]^{30}_D$ −32° ($CHCl_3$)]; UV, IR, $^1H$ and $^{13}C$ NMR, and EIMS data consistent with literature values.[22,25]

**(+)-Spiropachysine (6):** colorless needles; mp 278 °C [lit.[20] 278−280 °C]; $[\alpha]^{20}_D$ +34.8° (*c* 0.06, $CHCl_3$) [lit.[20] $[\alpha]^{22}_D$ +31.9° ($CHCl_3$)]; UV, IR, $^1H$ and $^{13}C$ NMR, and EIMS data consistent with literature values.[20]

**(+)-Axillaridine A (7):** colorless needles; mp 220−222 °C [lit.[21] 223−224 °C]; $[\alpha]^{20}_D$ +50.0° (*c* 0.06, $CHCl_3$) [lit.[21] $[\alpha]^{22}_D$ +51.3° ($CHCl_3$)]; UV, IR, $^1H$ and $^{13}C$ NMR, and EIMS data consistent with literature values.[21]

**(+)-Epipachysamine D (8):** colorless needles; mp 242−245 °C [lit.[18] 245−248 °C); $[\alpha]^{20}_D$ +19.8° (*c* 0.09, $CHCl_3$) [lit.[18] $[\alpha]^{30}_D$ +13° ($CHCl_3$)]; UV, IR, $^1H$ and $^{13}C$ NMR, and EIMS data consistent with literature values.[18,26]

**(+)-Pachysamine B (9):** colorless needles; mp 168 °C [lit.[19] 171−173 °C); $[\alpha]^{20}_D$ +48.9° (*c* 0.09, $CHCl_3$) [lit.[19] $[\alpha]^{30}_D$ +67° ($CHCl_3$)]; UV, IR, $^1H$ and $^{13}C$ NMR, and EIMS data consistent with literature values.[19,23]

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