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Isolation and Identification of Antiplatelet Aggregatory Principles from the Leaves of *Piper lolot*

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The methanolic extract of *Piper lolot*, having shown potent inhibitory activity on platelet aggregation induced by arachidonic acid (AA) and platelet activating factor (PAF), was subjected to activity-guided isolation to yield twelve new amide alkaloids, piperlotine A–L (**1**–**12**), along with twenty-nine known compounds. Their structures were elucidated on the basis of spectroscopic analysis. The isolated compounds were tested for their inhibitory activity on the rabbit platelet aggregation. The compounds piperlotine A (**1**), piperlotine C (**3**), piperlotine D (**4**), piperlotine E (**5**), 3-phenyl-1-(2,4,6-trihydroxyphenyl)propan-1-one (**21**), 3-(4-methoxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one (**22**), 1-*trans*-cinnamoylpyrrolidine (**24**), sarmentine (**26**), pellitorine (**27**), methyl 3-phenylpropionate (**32**), and (10*S*)-10-hydroxypheophorbide a methyl ester (**40**) showed potent antiplatelet aggregation activity.

KEYWORDS: ***Piper lolot*; Piperaceae; antiplatelet aggregation; piperlotine**

INTRODUCTION

Platelet aggregation plays a central role in thrombosis (clot formation). The presence of a thrombus in an artery providing blood to the heart is the most common cause of acute coronary syndromes such as myocardial infarction and angina. Inhibitors of aggregation can provide protection against these diseases and lower vascular disease mortality and stroke incidence in patients with unstable ischemic heart disease (*1*, *2*). Natural antithrombotic agents that influence platelet function are of potential interest for primary prevention of cardiovascular disease. In the course of our continuing search for novel antiplatelet aggregatory agents from natural sources (*3*–*6*), we found that the methanol extract of the leaves of *Piper lolot* displayed antiplatelet aggregation activity.

The genus *Piper* belongs to the Piperaceae family, widely distributed throughout the tropical and subtropical regions of the world, and encompasses over 700 species. Members of the *Piper* genus are of commercial, economical, and medicinal importance. Economically, the Piperaceae is employed for the production of pepper in worldwide spice markets. Plants from the genus *Piper* have been used for a number of practical applications, including remedies in many traditional medicinal systems, such as traditional Chinese medicine, the Indian Ayurvedic system, and folklore medicines of Latin America and the West Indies. *Piper* species have been extensively investigated as a source of new natural products with potential antitumoral, antimicrobial, antifungal, antiplatelet aggregation, and insecticidal activities (*7*–*13*). The phytochemical profile in *Piper* species is characterized by the production of typical classes of compounds such as amides, alkaloids, benzoic acids, lignans, neolignans, and a few flavones and chalcones (*9*–*12*, *14*–*18*). *P. lolot* is a small shrub found widely at lower elevations in Vietnam and often used to flavor meat in Southeast Asian dishes. It has been used to treat various diseases such as rheumatism, lumbago, digestive troubles, vomiting, diarrhea, and others (*13*, *15*). This species has not been the subject of thorough phytochemical analysis, and a methanolic extract of the leaves showed sufficient potent inhibitory activity on platelet aggregation induced by arachidonic acid (AA) and platelet activating factor (PAF) to warrant bioassay-guided fractionation.

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This led to the isolation of twelve hitherto undescribed amide derivatives (**1**–**12**) as well as twenty-nine known compounds. We describe herein the isolation, structural determination, and antiplatelet aggregation activity of isolated compounds.

MATERIALS AND METHODS

Equipment. Melting points were measured on a Yanagimoto MP-S3 micro melting point apparatus and are uncorrected. The UV spectra were recorded on a Hitachi UV-3210 spectrophotometer in MeOH solution. The IR spectra were measured on a Shimadzu FTIR-8501 spectrophotometer as KBr disks. The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian-400 Unity Plus spectrometer. Chemical shifts are shown in δ values with tetramethylsilane as an internal reference. The mass spectra were performed in the EI mode on a VG70-250S mass spectrometer.

Plant Material. The leaves of *Piper lolot* were collected from Vietnam in 2004 and verified by Prof. N. X. Dung. A voucher specimen (NXDUNG20040729) was deposited in the Herbarium of Hanoi National University, Hanoi, Vietnam.

Extraction and Separation. The leaves of *Piper lolot* (4.3 kg) were powdered and soaked with MeOH (5 L × 5) at room temperature, and the combined extracts were concentrated under reduced pressure to give a deep brown syrup (460 g). This was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer (95 g, after evaporation of the solvent) was directly chromatographed on a silica gel column by elution with a gradient of $CHCl_3$/Me_2CO to afford eleven fractions. Fraction 3 underwent column chromatographic separation over silica gel using *n*-hexane/EtOAc (19:1) as an eluent to yield **22** (17.2 mg) and **37** (1.1 mg). Fraction 5 was rechromatographed on a silica gel column and eluted with *n*-hexane/Me_2CO (9:1) to give **18** (2.1 mg), **19** (10.8 mg), **21** (7.2 mg), **23** (2.1 mg), **22** (426.5 mg), **32** (2.4 mg), **33** (1.1 mg), **34** (4.3 mg), and **38** (5.5 mg). Fraction 8 was chromatographed on silica gel and eluted with *n*-hexane/diisopropyl ether (2:1) to afford **26** (5.3 mg), **27** (6.2 mg), **31** (6.5 mg), **41** (7.3 mg), **5** (4.1 mg), **6** (5.3 mg), **7** (1.1 mg), and **8** (0.9 mg), successively. Fraction 9 underwent column chromatographic separation over silica gel using *n*-hexane/EtOAc (6:1) as an eluent to yield **13** (3.7 mg), **14** (2.1 mg), **15** (2.1 mg), **16** (0.4 mg), **24** (12.6 mg), **25** (1.1 mg), **1** (5.2 mg), **2** (1.3 mg), **30** (1.5 mg), **29** (0.7 mg), **3** (19.6 mg), **4** (2.3 mg), **9** (1.1 mg), and **12**(1.4 mg), successively. Fraction 11 was chromatographed on silica gel and eluted with $CHCl_3$/EtOAc (6:1) to afford **20** (8.9 mg), **17** (0.7 mg), **28** (0.6 mg), **40** (2.2 mg), **10** (0.9 mg), **11** (1.6 mg), **35** (0.7 mg), **39** (1.2 mg), and **36** (2.3 mg), successively.

Piperlotine-A (1). Colorless syrup. HREIMS *m/z* 231.1255 $[M]^+$ (calcd for $C_{14}H_{17}NO_2$, 231.1259). UV λ_{max} (MeOH) nm: 225, 300. IR ν (KBr) cm^{-1}: 828, 1030, 1173, 1250, 1439, 1511, 1600, 1647, 2955. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.59 (2H, dd, J = 7.2, 1.6 Hz, H-2′, 6′), 7.52 (1H, d, J = 15.2 Hz, H-β), 6.95 (2H, dd, J = 7.2, 1.6 Hz, H-3′, 5′), 6.82 (1H, d, J = 15.2 Hz, H-α), 3.82 (3H, s, 4′-OMe), 3.66 (2H, t, J = 6.4 Hz, H-2), 3.44 (2H, t, J = 6.8 Hz, H-5), 1.99 (2H, m, H-3), 1.85 (2H, m, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.7, 161.4, 141.9, 129.9, 128.6, 117.0, 114.7, 55.7, 46.9, 46.3, 26.4, 24.6. EIMS (% rel intensity), *m/z* 231 $[M]^+$ (35), 161 (100), 133 (20).

Piperlotine-B (2). Colorless syrup. HREIMS *m/z* 231.1263 $[M]^+$ (calcd for $C_{14}H_{17}NO_2$, 231.1259). UV λ_{max} (MeOH) nm: 215, 273. IR ν (KBr) cm^{-1}: 1029, 1173, 1254, 1444, 1511, 1603, 1638, 2920. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 (2H, d, J = 8.8 Hz, H-2′, 6′), 6.83 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.55 (1H, d, J = 12.4 Hz, H-β), 5.94 (1H, d, J = 12.4 Hz, H-α), 3.80 (3H, s, 4′-OMe), 3.52 (2H, t, J = 6.4 Hz, H-2), 3.22 (2H, t, J = 6.0 Hz, H-5), 1.83–1.74 (4H, m, H-3, 4). EIMS (% rel intensity), *m/z* 231 $[M]^+$ (42), 161 (100), 133 (18).

Piperlotine-A (3). White powder. Mp: 148–150 °C. HREIMS *m/z* 291.1473 $[M]^+$ (calcd for $C_{16}H_{21}NO_4$, 291.1470). UV λ_{max} (MeOH) nm: 231, 304. IR ν (KBr) cm^{-1}: 1007, 1125, 1332, 1418, 1452, 1505, 1584, 1647, 2968. ^{1}H NMR (400 MHz, acetone-d_6): δ 7.48 (1H, d, J = 15.2 Hz, H-β), 6.98 (2H, s, H-2′, 6′), 6.90 (1H, d, J = 15.2 Hz, H-α), 3.86 (6H, s, 3′-OMe, 5′-OMe), 3.74 (3H, s, 4′-OMe), 3.65 (2H, t, J = 6.8 Hz, H-2), 3.44 (2H, d, J = 6.8 Hz, H-5), 1.96 (2H, m, H-3), 1.85 (2H, m, H-4). ^{13}C NMR (100 MHz, acetone-d_6): δ 163.9, 153.9, 140.9, 139.9, 131.4, 119.3, 105.7, 59.9, 55.8, 46.3, 45.7, 26.1, 24.3. EIMS (% rel intensity), *m/z* 291 $[M]^+$ (43), 261 (41), 221 (86), 191 (100), 161 (43).

Piperlotine-D (4). Colorless syrup. HREIMS *m/z* 291.1466 $[M]^+$ (calcd for $C_{16}H_{21}NO_4$, 291.1470). UV λ_{max} (MeOH) nm: 226, 289. IR ν (KBr) cm^{-1}: 1005, 1124, 1330, 1417, 1582, 1648, 2942. ^{1}H NMR (400 MHz, acetone-d_6): δ 6.90 (2H, s, H-2′, 6′), 6.53 (1H, d, J = 12.4 Hz, H-β), 6.02 (1H, d, J = 12.4 Hz, H-α), 3.74 (6H, s, 3′-OMe, 5′-OMe), 3.72 (3H, s, 4′-OMe), 3.44 (2H, m, H-2), 3.30 (2H, m, H-5), 1.83 (4H, m, H-3, 4). EIMS (% rel intensity), *m/z* 291 $[M]^+$ (70), 221 (100), 191 (15).

Piperlotine-E (5). Colorless syrup. HREIMS *m/z* 215.0951 $[M]^+$ (calcd for $C_{13}H_{13}NO_2$, 215.0946). UV λ_{max} (MeOH) nm: 229, 235 (sh). IR ν (KBr) cm^{-1}: 742, 1224, 1465, 1513, 1710, 2926, 3355. ^{1}H NMR (400 MHz, acetone-d_6): δ 8.16 (1H, s, 4′-OH), 7.42 (2H, m, H-2, 5), 7.12 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.76 (2H, d, J = 8.4 Hz, H-3′, 5′), 6.26 (2H, m, H-3, 4), 3.20 (2H, t, J = 8.0 Hz, H-α), 2.95 (2H, t, J = 8.0 Hz, H-β). ^{13}C NMR (100 MHz, acetone-d_6): δ 170.2, 156.0, 131.6, 129.6, 119.2, 115.4, 112.7, 36.3, 29.5. EIMS (% rel intensity), *m/z* 215 $[M]^+$ (60), 148 (25), 120 (33), 107 (100).

Piperlotine-F (6). Colorless needles. Mp: 101–102 °C. HREIMS *m/z* 215.0941 $[M]^+$ (calcd for $C_{13}H_{13}NO_2$, 215.0946). UV λ_{max} (MeOH) nm: 211, 273. IR ν (KBr) cm^{-1}: 974, 1355, 1409, 1539, 1659, 1728, 2923. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (1H, d, J = 15.8 Hz, H-β), 7.84 (1H, d, J = 15.8 Hz, H-α), 7.61 (2H, m, H-2′, 6′), 7.38 (3H, m, H-3′, 4′, 5′), 3.93 (2H, t, J = 7.2 Hz, H-5), 2.66 (2H, t, J= 6.8 Hz, H-3), 2.08 (2H, m, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.7, 166.3, 144.0, 134.4, 129.2, 128.3, 128.3, 119.0, 45.8, 33.6, 17.2. EIMS (% rel intensity), *m/z* 215 $[M]^+$ (7), 149 (29), 131 (100), 103 (39).

Piperlotine-G (7). Colorless needles. Mp: 140–142 °C. HREIMS *m/z* 245.1048 $[M]^+$ (calcd for $C_{14}H_{15}NO_3$, 245.1052). UV λ_{max} (MeOH) nm: 222, 284. IR ν (KBr) cm^{-1}: 1025, 1177, 1248, 1348, 1514, 1600, 1662, 1730, 2936. ^{1}H NMR (400 MHz, acetone-d_6): δ 7.85 (1H, d, J = 16.0 Hz, H-β), 7.71 (1H, d, J = 16.0 Hz, H-α), 7.69 (2H, d, J = 7.2 Hz, H-2′, 6′), 7.00 (2H, d, J = 7.2 Hz, H-3′, 5′), 3.85 (3H, s, 4′-OMe), 3.82 (2H, m, H-5), 2.61 (2H, m, H-3), 2.06 (2H, m, H-4). EIMS (% rel intensity), *m/z* 245 $[M]^+$ (44), 161 (100), 133 (17).

Piperlotine-H (8). Colorless syrup. HREIMS *m/z* 245.1050 $[M]^+$ (calcd for $C_{14}H_{15}NO_3$, 245.1052). UV λ_{max} (MeOH) nm: 224, 274. IR ν (KBr) cm^{-1}: 1030, 1176, 1252, 1350, 1601, 1658, 2923. ^{1}H NMR (400 MHz, acetone-d_6): δ 7.61 (2H, d, J = 8.0 Hz, H-2′, 6′), 6.87 (2H, d, J = 8.0 Hz, H-3′, 5′), 6.85 (1H, d, J = 12.8 Hz, H-β), 6.80 (1H, d, J = 12.8 Hz, H-α), 3.82 (2H, m, H-5), 3.82 (3H, s, 4′-OMe), 2.57 (2H, m, H-3), 2.06 (2H, m, H-4). EIMS (% rel intensity), *m/z* 245 $[M]^+$ (33), 178 (15), 161 (75), 153 (44), 136 (39), 107 (53), 77 (100).

Piperlotine-I (9). Colorless syrup. [α]: +23.2 (MeOH; *c* 0.08). HREIMS *m/z* 231.1255 $[M]^+$ (calcd for $C_{14}H_{17}NO_2$, 231.1259). UV λ_{max} (MeOH) nm: 210, 216, 222, 274. IR ν (KBr) cm^{-1}: 1052, 1129, 1341, 1449, 1548, 1657, 2930. ^{1}H NMR (400 MHz, acetone-d_6): δ 7.56 (2H, m, H-2′, 6′), 7.52 (1H, d, J = 15.6 Hz, H-β), 7.39 (3H, m, H-3′, 4′, 5′), 6.65 (1H, d, J = 15.6 Hz, H-α), 4.37 (1H, t, J = 5.2 Hz, H-2), 3.30 (2H, m, H-5), 3.26 (3H, s, 2-OMe), 1.80 (4H, m, H-3, 4). ^{13}C NMR (100 MHz, acetone-d_6): δ 164.4, 139.2, 135.7, 129.4, 129.0, 127.7, 122.4, 104.4, 52.2, 39.0, 30.1, 25.0. EIMS (% rel intensity), *m/z* 231 $[M]^+$ (11), 131 (100), 103 (32), 77 (23).

Piperlotine-J (10). Colorless syrup. HREIMS *m/z* 277.1310 $[M]^+$ (calcd for $C_{15}H_{19}NO_4$, 277.1314). UV λ_{max} (MeOH) nm: 212, 218, 223, 280. IR ν (KBr) cm^{-1}: 977, 1236, 1429, 1587, 1645, 1733, 2928, 3371. ^{1}H NMR (400 MHz, CD_3OD): δ 7.62 (2H, m, H-2′, 6′), 7.60 (1H, d, J = 15.6 Hz, H-β), 7.39 (3H, m, H-3′, 4′, 5′), 6.96 (1H, d, J = 15.6 Hz, H-α), 5.40 (1H, m, H-3), 3.95 (2H, m, H-1), 3.85 (2H, m, H-4), 2.17 (2H, m, H-2), 2.05 (3H, s, OAc). ^{13}C NMR (100 MHz, CD_3OD): δ 171.0, 166.1, 142.6, 135.1, 129.8, 128.8, 128.0, 118.0, 74.1, 52.3, 44.7, 29.7, 19.7. The enantiomer: ^{1}H NMR (400 MHz, CD_3OD): δ 7.62 (2H, m, H-2′, 6′), 7.60 (1H, d, J = 15.6 Hz, H-β), 7.39 (3H, m, H-3′, 4′, 5′), 6.88 (1H, d, J = 15.6 Hz, H-α), 5.33 (1H, m, H-3), 3.75 (2H, m, H-4), 3.58 (2H, m, H-1), 2.27 (2H, m, H-2), 2.04 (3H, s, OAc). ^{13}C NMR (100 MHz, CD_3OD): δ 171.0, 166.1, 142.6, 135.1, 129.8, 128.8, 128.0, 118.1, 72.7, 51.8, 44.1, 31.3, 19.7. EIMS (% rel intensity), *m/z* 217 $[M - AcOH]^+$ (40), 199 (10), 131 (100), 103 (37), 77 (19).

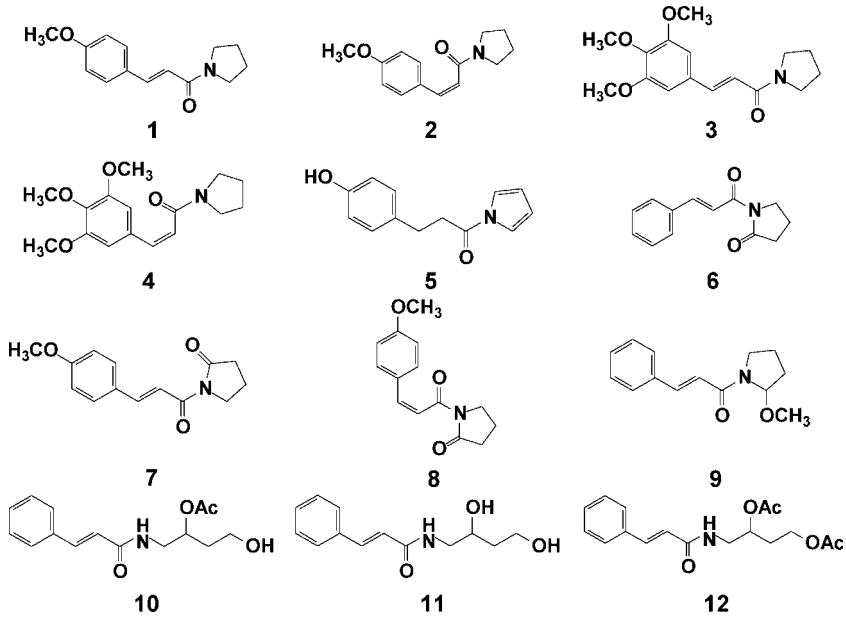

Figure 1

Piperlotine-K (11). Colorless syrup. HREIMS *m/z* 235.1214 [M^+] (calcd for $C_{13}H_{17}NO_3$, 235.1208). UV λ_{max} (MeOH) nm: 211, 218, 224, 280. IR ν (KBr) cm^{-1}: 976, 1103, 1438, 1580, 1593, 1647, 2947, 3380. ^1H NMR (400 MHz, acetone-d_6): δ 7.64 (2H, m, H-2′, 6′), 7.58 (1H, d, $J = 15.6$ Hz, H-β), 7.39 (3H, m, H-3′, 4′, 5′), 6.99 (1H, d, $J = 15.6$ Hz, H-α), 4.54 (1H, m, H-3), 3.82 (2H, m, H-1), 3.66 (2H, m, H-4), 2.04 (2H, m, H-2). ^{13}C NMR (100 MHz, acetone-d_6): δ 164.4, 141.0, 135.8, 129.6, 129.0, 128.1, 119.8, 70.7, 54.8, 44.5, 34.5. The enantiomer: ^1H NMR (400 MHz, acetone-d_6): δ 7.64 (2H, m, H-2′, 6′), 7.58 (1H, d, $J = 15.6$ Hz, H-β), 7.39 (3H, m, H-3′, 4′, 5′), 6.94 (1H, d, $J = 15.6$ Hz, H-α), 4.44 (1H, m, H-3), 3.57 (4H, m, H-1, 4), 1.93 (2H, m, H-2). ^{13}C NMR (100 MHz, acetone-d_6): δ 164.4, 141.0, 135.8, 129.6, 129.0, 128.1, 120.0, 69.0, 54.4, 44.0, 32.9. EIMS (% rel intensity), *m/z* 217 [$M - H_2O]^+$ (65), 131 (100), 103 (45), 77 (22).

Piperlotine-L (12). Colorless syrup. [α]: +42.2 (MeOH; *c* 0.1). HREIMS *m/z* 319.1418 [M^+] (calcd for $C_{17}H_{21}NO_5$, 319.1420). UV λ_{max} (MeOH) nm: 210, 216, 222, 274. IR ν (KBr) cm^{-1}: 979, 1043, 1232, 1547, 1621, 1660, 1736, 2935, 3283. ^1H NMR (400 MHz, $CDCl_3$): δ 7.62 (1H, d, $J = 15.6$ Hz, H-β), 7.51 (2H, m, H-2′, 6′), 7.38 (3H, m, H-3′, 4′, 5′), 6.39 (1H, d, $J = 15.6$ Hz, H-α), 5.98 (1H, br, NH), 5.11 (1H, m, H-3), 4.15 (2H, m, H-4), 3.62 (2H, m, H-1), 2.10 (3H, s, OAc), 2.06 (3H, s, OAc), 1.96 (2H, m, H-2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.4, 171.2, 166.3, 141.9, 134.9, 130.0, 129.1, 128.1, 120.3, 70.7, 60.5, 43.4, 31.2, 21.3, 21.1. EIMS (% rel intensity), *m/z* 319 $[M]^+$ (1), 199 (21), 161 (27), 131 (100), 103 (25), 77 (11).

Preparation of the Platelet Suspension. Washed platelet suspension was prepared as previously described with some modifications (*19–21*). In brief, blood was collected from the marginal ear vein of New Zealand White rabbits into tubes containing one-sixth volume of acid-citrate-dextrose as anticoagulant. The blood was centrifuged at 1000*g* for 8 min at room temperature. The upper portion was kept as platelet-rich plasma (PRP) after mixing with EDTA to a final concentration of 5 mM and recentrifuged at 2000*g* for 12 min. The platelet pellet was suspended in modified Ca^{2+}-free Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES) with 0.35% bovine serum albumin, heparin (50 unit/mL), and apyrase (1 unit/mL) and then was incubated at 37 °C for 20 min. After centrifugation at 2000*g* for 6 min, the washed platelet pellet was resuspended in Tyrode's buffer containing 1 mM Ca^{2+}. For the aggregation test, the platelet numbers were counted by hemacytometer and adjusted to 2.5×10^8 platelets/mL.

Measurement of Platelet Aggregation. Platelet aggregation was measured turbidimetrically with a light-transmission Platelet Aggregation Chromogenic Kinetic System PACK4 (Helena Laboratories, Beaumont TX) with some modifications (*19–21*). The platelet suspension was stirred at 900 rpm and incubated with an appropriate amount of vehicle (dimethyl sulfoxide, DMSO) or various concentrations of test compounds in DMSO at 37 °C for 2 min. Aggregation was induced with PAF (5 nM) or AA (100 μM). The transmission of washed platelet suspension was assigned 0% aggregation while transmission through Tyrode's buffer was assigned 100% aggregation. The extent of platelet aggregation was measured as the maximal increase in light transmission within 4 min after the addition of an inducer. To eliminate or minimize any possible effects of the solvent, the final concentration of DMSO in the platelet suspension was fixed at 0.5%.

RESULTS AND DISCUSSION

Extraction of the leaves of *P. lolot* with MeOH followed by liquid–liquid partition resulted in the localization of the anti-platelet activity in the chloroform fraction. Further fractionation on a silica gel column yielded fractions rich in a mixture of amide derivatives. These fractions were subjected to further chemical analysis to give twelve amide derivatives (**1**–**12**) (**Figure 1**) and twenty-nine known compounds.

Piperlotine-A (**1**) was isolated as colorless syrup and had a HREIMS molecular ion peak indicating a molecular formula of $C_{14}H_{17}NO_2$. The UV absorption maxima at 225 and 300 nm coupled with the IR bands at 1647 cm^{-1} indicated the presence of an *E*-cinnamoyl amide system. In the ^1H NMR of **1**, AB type proton signals at δ 7.59 (1H, dd, $J = 7.2$, 1.6 Hz, H-2′/6′) and 6.95 (2H, dd, $J = 7.2$, 1.6 Hz, H-3′/5′), conjugated *trans* double bond proton signals at δ 7.52 and 6.82 (each 1H, d, $J = 15.2$ Hz), and a methoxyl signal at δ 3.82 (3H, s) were consistent with a *p*-methoxy-*E*-cinnamoyl moiety. Additional signals for the presence of a pyrrolidine moiety were indicated by signals of the four mutually coupled methylene groups at δ 3.66 (2H, t, $J = 6.4$ Hz, H-2), 3.44 (2H, t, $J = 6.8$ Hz, H-5), 1.99 (2H, m, H-3), and 1.85 (2H, m, H-4). With the basic fragments of **1** established, the connectivities between them were solved by the use of HMBC and NOESY correlations. On the basis of the above evidence, the structure of **1** was assigned as (4-methoxy-*E*-cinnamoyl)pyrrolidine. To the best of our knowledge, this is the first report of (4-methoxy-*E*-cinnamoyl)pyrrolidine from a natural source. However, the title compound has been prepared during the synthesis of its diaziridine derivative by Ishihara et al. (*22*).

Piperlotine-B (**2**), isolated as a colorless syrup, showed the same molecular formula of $C_{14}H_{17}NO_2$ as **1** by HREIMS. When

comparing the ^{1}H NMR spectrum of **2** with that of **1**, the signals were superimposable except for the signals due to H-α and H-β, which suggested these two compounds may be geometrical isomers sharing the same structural features. The signals due to H-α and H-β of the conjugated carbonyl system resonated at δ 6.55 (1H, d, J = 12.4 Hz, H-β) and 5.94 (1H, d, J = 12.4 Hz, H-α). The coupling constant indicated that the double bond possesses *Z* geometry. The attribution of this configuration was corroborated by the shielded signals of H-2′/6′ and H-3′/5′ and fewer UV absorption maxima in the *Z*-isomer (273 nm) when compared with the *E*-isomer (300 nm). Thus, the structure of **2** was determined as (4-methoxy-*Z*-cinnamoyl)pyrrolidine.

Piperlotine-C (**3**) was obtained as a white powder with a molecular formula of $C_{16}H_{21}NO_4$ by HREIMS. The IR spectrum of **3** showed bands at 1647 cm^{-1} (conjugated carbonyl group) and 1505, 1584 cm^{-1} (aromatic ring). The ^{1}H NMR spectrum of **3** showed typical signals for a pyrrolidine ring at δ 3.65 (2H, t, J = 6.8 Hz, H-2), 3.44 (2H, d, J = 6.8 Hz, H-5), 1.96 (2H, m, H-3), and 1.85 (2H, m, H-4). The ^{1}H NMR spectrum also displayed signals characteristic of a trimethoxy-*E*-cinnamoyl moiety. These consisted of *trans* coupled olefinic protons at δ 7.48 (1H, d, J = 15.2 Hz) and 6.90 (1H, d, J = 15.2 Hz) for the conjugated carbonyl system; signals for three methoxyl groups at δ 3.86 (6H, s) and 3.74 (3H, s), two of which are equivalent; and a shielded aromatic singlet integrating for two protons of a symmetrically substituted aromatic ring at δ 6.98 (2H, s, H-2′/6′). The corresponding carbon signals were assigned with the aid of the HMQC spectra. The substitution pattern of the aromatic ring and the connection between the previously mentioned two moieties were confirmed by the correlations observed in the NOESY and HMBC spectra. Therefore, the structure of **3** was established as *N*-(trimethoxy-*E*-cinnamoyl)pyrrolidine. Although **3** was reported as a synthetic product (*23*), this is the first report of its occurrence in nature.

Piperlotine-D (**4**) was obtained as a colorless syrup and shown to have a molecular formula of $C_{16}H_{21}NO_4$. All the spectra of **4** were similar to those of **3** and suggested that it is an isomer of **3**. The most obvious difference between the ^{1}H NMR spectra resulted from the presence of signals for an α,β-unsaturated carbonyl system with a *Z*-configuration in **4** at δ 6.53 (1H, d, J = 12.4 Hz) and 6.02 (1H, d, J = 12.4 Hz), instead of an *E*-configuration in **3**. Thus, **4** was identified as *N*-(trimethoxy-*Z*-cinnamoyl)pyrrolidine. Bruening et al. (*24*) have synthesized this compound, but this is the first report as a natural product.

Piperlotine-E (**5**) was obtained as a colorless syrup. The molecular formula of **5** was established as $C_{13}H_{13}NO_2$ by HREIMS. Its UV absorption maxima at 229 and 235 (sh) nm were consistent with an aromatic compound. The IR absorption bands at 3355, 1710, and 1513, 1465 cm^{-1} indicated the presence of a hydroxyl, a conjugated carbonyl group of an amide, and an aromatic ring. The ^{1}H NMR spectrum of **5** displayed typical signals corresponding to a *p*-hydroxyphenyl propanoyl moiety. These contained AB type signals at δ 7.12 (2H, d, J = 8.4 Hz, H2′/H-6′) and 6.76 (2H, d, J = 8.4 Hz, H-3′/H-5′), a hydroxyl group at δ 8.16 (1H, s), and coupled triplets for two methylenes at δ 3.20 (2H, t, J = 8.0 Hz, H-α) and 2.95 (2H, t, J = 8.0 Hz, H-β). The carbon signals at δ > 170.2 (C═O), 36.3 (C-α), and 29.5 (C-β) corroborated the presence of a propanoyl moiety in **5**. In addition, a set of signals for a pyrrole ring were also observed at δ 7.42 (2H, m, H-2, 5) and 6.26 (2H, m, H-3, 4). Analysis of HMQC, COSY, and HMBC data enabled the complete assignment of the signals for this compound, leading to its formulation as *N*-(*p*-hydroxyphenylpropanoyl)pyrrole.

Piperlotine-F (**6**) was obtained as colorless needles, mp 101–102 °C. It exhibited a molecular formula of $C_{13}H_{13}NO_2$, on the basis of its HREIMS data. Its UV absorption maxima at 211 and 273 nm indicated the presence of a cinnamoyl chromophore in the molecule. The IR spectrum showed absorption bands corresponding to conjugated amide carbonyl (1659 cm^{-1}), a γ-lactam (1728 cm^{-1}), and an aromatic ring (1409, 1539 cm^{-1}). A *trans* cinnamoyl moiety was apparent from the NMR signals at δ_H 7.61 (2H, m) and 7.38 (3H, m), attributable to H-2′, 6′ and H-3′, 4′, 5′, respectively, and *trans* coupled olefinic protons at δ_H 7.95 and 7.84 (each 1H, d, J = 15.8 Hz), together with the carbon signals at δ_C 166.3 (C═O), 144.0 (C-β), and 119.0 (C-α). In addition, a set of signals for a pyrrolidin-2-one residue were also observed at δ_H 3.93 (2H, t, J = 7.2 Hz, H-5), 2.66 (2H, t, J = 6.8 Hz, H-3), and 2.08 (2H, m, H-4), and related carbons were observed at δ_C 175.7, 45.8, 33.6, and 17.2 ppm. The carbonyl at C-2 was supported by the unusual downfield shift of the H-α signal to δ_H 7.84. Connectivities between these two moieties were determined with the aid of an HMBC experiment. Finally, the structure of **6** was deduced as *N*-(*E*-cinnamoyl)pyrrolidin-2-one, which has been synthesized by Soloshonok et al. (*25*).

Piperlotine-G (**7**) was isolated as a white powder, mp 140–142 °C, and shown to have a molecular formula of $C_{14}H_{15}NO_3$ on the basis of HREIMS. Its UV and IR spectra were consistent with the presence of a cinnamoyl chromophore. In the ^{1}H NMR spectrum, AB type aromatic proton signals at δ 7.69 (2H, d, J = 7.2 Hz, H-2′, 6′) and 7.00 (2H, d, J = 7.2 Hz, H-3′, 5′) and a methoxyl signal at δ 3.85 (3H, s), together with the *trans* coupled proton signals at δ 7.85 and 7.71 (each 1H, d, J = 16.0 Hz), indicated the presence of the *p*-methoxycinnamoyl moiety in **7**. Besides this moiety, the ^{1}H NMR spectrum also showed signals due to a pyrrolidin-2-one ring at δ 3.82 (2H, m, H-5), 2.61 (2H, m, H-3), and 2.06 (2H, m, H-4). These data were in agreement with those reported for the synthetic sample prepared by Sibi et al. (*26*). Thus, **7** was identified as *N*-(*p*-methoxy-*E*-cinnamoyl)pyrrolidin-2-one, and this is the first report from the natural source.

Piperlotine-H (**8**) was obtained as a colorless syrup. HREIMS data of this compound corresponded to a molecular formula of $C_{14}H_{15}NO_3$, as in **7**, indicating it to be a structural isomer. Compound **8** and **7** were found to have similar structures by comparison of their UV, IR, and NMR spectra. The observed difference was the appearance of *cis* coupled olefinic protons at δ 6.85 and 6.80 (each 1H, d, J = 12.8 Hz), which indicated that compound **8** is a *Z*-isomer of **7**. Therefore, the structure of **8** was assigned as *N*-(*p*-methoxy-*Z*-cinnamoyl)pyrrolidin-2-one.

Piperlotine-I (**9**) was obtained as a colorless syrup. The HREIMS of **9** was consistent with a molecular formula of $C_{14}H_{17}NO_2$. The UV absorption maxima at 274 nm suggested the presence of a cinnamoyl residue. In the IR spectrum, bands at 1657 and 1449, 1548 cm^{-1} revealed the presence of conjugated amide carbonyl group and an aromatic ring. Accordingly, the ^{1}H NMR spectrum displayed characteristic signals for a cinnamoyl group (δ 7.56, 2H, m; 7.39, 3H, m; 7.52 and 6.65, each 1H, d, J = 15.6 Hz). A methoxyl singlet at δ 3.26 and the signals at δ 4.37 (1H, t, J = 5.2 Hz, H-2), 3.30 (2H, m, H-5), and 1.80 (4H, m, H-3, 4), in addition to the carbon signals in the ^{13}C NMR spectrum at δ 52.2 and 104.4, 39.0, 30.1, and 25.0, suggested the presence of a 2-methoxypyrrolidine residue. These structural fragments were confirmed by the analysis of ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. The downfield

shift of C-2 to δ_C 104.4 suggested that the methoxyl group was located at C-2 of the pyrrolidine ring. This was further supported by a ^3J correlation between OMe (δ_H 3.26) and C-2 (δ_C 104.4) in the HMBC experiment. Analysis of all the available data led us to conclude that **9** is *N*-cinnamoyl-2-methoxypyrrolidine.

Piperlotine-J (**10**) was obtained as a racemic mixture. The HREIMS data corresponded to the molecular formula $C_{15}H_{19}NO_4$. The UV spectrum of **10** showed absorption at 280 nm, indicating it to be an aromatic compound. The IR bands at 3371, 1733, and 1645 cm^{-1} were consistent with the presence of hydroxyl, ester carbonyl, and conjugated amide carbonyl groups. The ^1H NMR spectrum displayed signals for five aromatic protons (δ 7.62 and 7.39) and a pair of olefinic protons (δ 7.60 and 6.96, $J = 15.6$ Hz), attributable to a cinnamoyl moiety. A set of mutually coupled protons deduced with the aid of ^1H–^1H COSY at δ 5.40 (1H, m, H-3), 3.95 (2H, m, H-1), 3.85 (2H, m, H-4), and 2.17 (2H, m, H-2) suggested the presence of a 3,4-disubstituted butanol moiety. The NMR spectra showed signals for an acetoxyl group at δ_H 2.05 and δ_C 19.7 and 171.0, the position of which was located at C-3, due to a ^3J HMBC correlation from H-3 (δ_H 5.40) to the acetyl carbonyl (δ_C 171.0), and a downfield shift of H-3 to δ_H 5.40. The HMBC spectrum of **10** also showed a ^3J correlation from H-4 (δ_H 3.85) to the amide carbonyl carbon at δ_C 166.1, which indicated that the cinnamoyl moiety was attached to C-4 of the 3-acetoxybutan-1-ol unit by an amide linkage. Thus, the structure of **10** was deduced to be 4-*N*-cinnamoyl-3-acetoxylbutanol. The other set of signals assignable to a 4-substituted 3-acetoxylbutanol side chain of an enantiomer of **10** appeared at δ_H 5.33 (1H, m, H-3), 3.75 (2H, m, H-4), 3.58 (2H, m, H-1), 2.27 (2H, m, H-2), and 2.04 (3H, s, OAc).

Piperlotine-K (**11**) was also obtained as a racemic mixture. It was deduced to have an elemental composition of $C_{13}H_{17}NO_3$ from its HREIMS data. The UV spectrum was similar to that of **10**, and the IR absorption bands at 3380 and 1647 cm^{-1} indicated the presence of hydroxyl and conjugated amide functionalities, respectively. The NMR spectra were similar to those of **10**, except for the lack of signals for the acetyl group. Thus, as in the case of **10**, the NMR data including COSY and HMBC information were consistent with the presence in **11** of an *E*-cinnamoyl amide unit (δ_H 7.64, m, 2H; 7.39, m, 3H; 7.58 and 6.99, each 1H, d, $J = 15.6$ Hz) linked through an amide bond with C-4 of a 3,4-disubstituted butanol moiety (δ_H 4.54, 1H, m, H-3; 3.82, 2H, m, H-1; 3.66, 2H, m, H-4; 2.04, 2H, m, H-2). However, **11** possesses at C-3 a hydroxyl group instead of the acetyl grouping of **10**, which was strongly supported by the upfield shift of H-3 to δ_H 4.54. Thus, the structure of **11** was elucidated as 4-*N*-*E*-cinnamoylbutane-1,3-diol. The other set of signals due to the 4-substituted butan-1,3-diol moiety of an enantiomer of **11** appeared at δ_H 4.44 (1H, m, H-3), 3.57 (4H, m, H-1, 4), and 1.93 (2H, m, H-2).

Piperlotine-L (**12**) was obtained as a colorless syrup with an elemental composition of $C_{17}H_{21}NO_5$, as determined from its HREIMS. The UV absorption maxima at 274 nm and the IR bands at 3283, 1736, and 1660 cm^{-1} were similar to those of **10** and **11**. The ^1H spectra revealed signals due to the *trans*-cinnamoyl amide moiety (δ_H 7.51, 2H, m, H-2′, 6′; 7.38, 3H, m, H-3′, 4′, 5′; 7.62 and 6.39, each 1H, d, $J = 15.6$ Hz, H-α, -β; 5.98, 1H, br, NH) and the 4-substituted-1,3-dioxygenated butane chain (δ_H 5.11, 1H, m, H-3; 4.15, 2H, m, H-4; 3.62, 2H, m, H-1; 1.96, 2H, m, H-2). These data were similar to those of **11**, except for the presence of two acetyl groups that resonated at δ_H 2.10, 2.06 (each 3H, s) and δ_C 171.4, 171.2, 21.3, 21.1. These two acetyl groups were placed at C-1 and C-3 on the basis of the low-field shifts of H-1 to δ_H 3.62 and H-3 to δ_H 5.11, and they were assigned by the COSY, HMQC, and HMBC spectra. The HMBC spectrum also confirmed the connectivity of the above two spin systems through an amide linkage at C-4. Finally, the structure of **12** was elucidated as 4-*N*-*E*-cinnamoyl-1,3-diacetoxybutane.

Table 1. Effect of Principles from *Piper lolot* on the Platelet Aggregation Induced by Arachidonic Acid (AA) and Platelet Activating Factor (PAF)[a]

	anti-AA		anti-PAF	
compd	inhibition (%) at 100 μg/mL	IC_{50} (μg/mL)	inhibition (%) at 100 μg/mL	IC_{50} (μg/mL)
1	100.0	15.2 ± 3.0	13.0	
3	100.0	26.6 ± 5.1	29.5	
4	100.0	43.4 ± 5.4	2.1	
5	96.2	11.5 ± 4.6	74.7	58.6 ± 1.4
6	0.9		3.5	
12	−1.2		7.7	
14	2.2		−0.7	
18	6.8		7.9	
20	5.3		3.3	
21	100.0	19.0 ± 6.0	95.4	61.0 ± 2.5
22	100.0	31.2 ± 1.1	97.7	71.4 ± 7.5
24	100.0	7.3 ± 0.2	28.5	
26	100.0	49.4 ± 3.4	97.1	52.6 ± 2.7
27	87.4	53.0 ± 1.8	25.5	
31	−1.6		−5.3	
32	100.0	53.4 ± 1.5	67.0	83.2 ± 1.3
34	0.8		−4.1	
38	1.2		2.3	
40	77.4	38.1 ± 3.2	78.9	50.3 ± 5.3
Aspirin[b]	100.0	5.5 ± 0.9		
CV3988[b]			100.0	1.5 ± 0.3

[a] The antiplatelet aggregation (%) was calculated by the following equation: antiplatelet aggregation (%) = [1 − (platelet aggregation potency of sample/platelet aggregation potency of vehicle)] × 100%. The IC_{50} value of each principle was calculated and shown as mean ± SD ($n = 4$–6). [b] Positive control: CV3988 [3-(*N*-octadecylcarbamoyl)-2-methoxypropyl(2-thiazolioethyl) phosphate], a specific PAF receptor antagonist (*41*).

In addition to these twelve new compounds, twenty-nine known compounds including five phenanthrene type alkaloids [cepharadione A (**13**) (*16*), cepharanone B (**14**) (*16*), piperolactam A (**15**) (*16*), aristololactam A-II (**16**) (*27*), and norariستolodione (**17**) (*28*)], three sterols [β-sitosterol (**18**) (*29*), stigmasterol (**19**) (*29*), and stigmasterol glucoside (**20**) (*29*)], three chalcones [3-phenyl-1-(2,4,6-trihydroxyphenyl)propan-1-one (**21**) (*10*), 3-(4-methoxyphenyl)-1-(2,4,6-trihydroxyphenyl) propan-1-one (**22**) (*10*), and 2′,4′,6′-trihydroxychalcone (**23**) (*30*)], five amides [1-*trans*-cinnamoylpyrrolidine (**24**) (*16*), 1-*cis*-cinnamoylpyrrolidine (**25**) (*31*), sarmentine (**26**) (*11*), pellitorine (**27**) (*12*), and tyraminylferulamide (**28**) (*16*)], six benzenoids [methylparaben (**29**) (*32*), vanillic acid (**30**) (*32*), hydrocinnamic acid (**31**) (*33*), methyl 3-phenylpropionate (**32**) (*34*), methyl 3-(4-hydroxyphenyl)propionate (**33**) (*10*), and 3-(4-methoxyphenyl)propionic acid methyl ester (**34**) (*35*)], two ionones [dehydrovomifoliol (**35**) (*36*) and 5,6-epoxy-3-hydroxy-7-megastigmene-9-one (**36**) (*37*)], demethoxyyangonin (**37**) (*17*), *trans*-nerolidol (**38**) (*18*), loliolide (**39**) (*38*), (10*S*)-10-hydroxypheophorbide a methyl ester (**40**) (*39*), and melissic acid (**41**) (*40*) were isolated. The structures of these known compounds were identified by spectroscopic analyses and/or by comparison with data reported in the literature.

Nineteen compounds obtained from this study were evaluated for their antiplatelet aggregation activities. As shown in **Table 1**, compounds **1**, **3**, **4**, **5**, **21**, **22**, **24**, **26**, **27**, **32**, and **40** showed antiplatelet aggregation activity. At 100 μg/mL, compounds **1**,

3, **4**, **21**, **22**, **24**, **26**, and **32** showed 100% inhibition of platelet aggregation induced by arachidonic acid. Among them, compound **24** is the most active inhibitor of platelet aggregation with an IC_{50} of 7.3 μg/mL, comparable with that of aspirin (IC_{50} 5.5 μg/mL), a clinically used antiplatelet aggregatory agent. Isolates **1**, **3**, **5**, **21**, and **22** also exhibited strong antiplatelet aggregation activity with the IC_{50} values of 15.2, 26.6, 11.5, 19.0, and 31.2 μg/mL, respectively. Like aspirin, compounds **1**, **3**, **4**, **24**, and **27** were more selective inhibitors of the platelet aggregation induced by arachidonic acid. Among those tested, compounds **21**, **22**, and **26** displayed more than 95% inhibition against platelet aggregation induced by PAF, whereas compounds **5** and **40** showed more than 75% inhibition at the concentration of 100 μg/mL. Among all these, **40** is the most active compound with an IC_{50} value of 50.3 μg/mL. The amides containing a pyrrole or pyrrolidine ring (**1**, **3**, **4**, **5**, and **24**) are more active than other compounds, suggesting that the five member ring is important for antiplatelet aggregation induced by arachidonic acid.

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