Two Novel Cucurbitacins, Neocucurbitacins A and B, from the Brazilian Folk Medicine "Buchinha" (*Luffa operculata*) and Their Effect on PEBP2αA and OCIF Gene Expression in a Human Osteoblast-Like Saos-2 Cell Line

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Received May 30, 2001; accepted August 1, 2001

Two novel cucurbitacins designated as neocucurbitacins A (1), possessing inhibitory activity of polyoma enhancer binding protein 2αA (PEBP2αA) and osteoclastogenesis-inhibitory factor (OCIF) gene expression in human osteoblast-like cells, and B (2) were isolated from the fruit of *Luffa operculata.* Their structures have been determined by extensive spectroscopic investigation.

Key words neocucurbitacin; *Luffa operculata*; Buchinha; Cucurbitaceae; cucurbitacin; bone formation

Bone mass is regulated coordinately by bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). Using an RT-PCR-based bioassay[1] with human Saos-2 osteoblast-like cells, we have been searching for chemicals that can modulate the gene expression of polyoma enhancer binding protein 2αA (PEBP2αA) and osteoclastogenesis-inhibitory factor (OCIF), which play important roles in bone metabolism. PEBP2αA/AML3/CBFA1 is one transcription regulator of osteoblast differentiation, and knockout mice in which the PEBP2αA gene was mutated showed no osteogenesis.[2,3] OCIF[4]/OPG[5] is a protein secreted from osteoblasts or stromal cells and acts as a decoy receptor for osteoclast differentiation factor (ODF),[6,7] which mediates an essential signal to osteoclast precursors for their differentiation into osteoclasts. OCIF-deficient mice exhibit severe osteoporosis.[8,9] Our aim in the screening work is to obtain natural compounds that can be used as probes for pharmacological studies on the intracellular signal transduction pathway directing the gene expression of PEBP2αA and OCIF.

Screening of medicinal plants from South America by the assay using Saos-2 cells[1] indicated that an MeOH extract of *Luffa operculata* (Cucurbitaceae), a biennial medicinal plant growing in the Amazon region, inhibited the gene expression of both PEBP2αA and OCIF. The plant is commonly known as Buchinha or Bucha-dos-paulistas in Brazil, and the aqueous extract of the fruit has been used as a remedy for urethritis and empyema, and in treatment of edema.[10] Bioassay-guided purification of a crude extract of *L. operculata* led to the isolation of two novel cucurbitacins designated as neocucurbitacins A (**1**) and B (**2**), along with cucurbitacins B (**3**), D (**4**), E (**5**),[11] and isocucurbitacin B (**6**).[12] The structural elucidation of the above compounds **1** and **2** are reported in this communication.

The MeOH extract (16.35 g) of the fruit of *L. operculata* (200 g) was dissolved in CH_2Cl_2. The CH_2Cl_2-soluble fraction (8.52 g) was subjected to silica gel column chromatography using a $CHCl_3$–MeOH (50 : 1) solvent system, followed by HPLC (Hibar LiChroCART, 10×250 mm, Merck Co.) with $CHCl_3$ alone to yield **1** (8 mg). Using a $CHCl_3$–MeOH (10 : 1) solvent system, followed by HPLC (Hibar LiChroCART, 10×250 mm, Merck Co.) with $CHCl_3$–MeOH (20 : 1) also yielded **2** (7 mg).

Compound **1**, in the form of a colorless amorphous powder, $[\alpha]_D$ +71.3° (c=0.46, $CHCl_3$), gave a molecular ion at m/z 542 $(M)^+$ in electron-impact (EI)-MS, and high-resolution (HR)-EI-MS determined the molecular formula to be $C_{31}H_{42}O_8$ ($[M]^+$ 542.2877, Calcd 542.2880). The IR (3460, 1763, 1736, 1697 cm^{-1}) spectrum suggested the presence of hydroxyl groups and carbonyl groups. The ^{1}H-NMR[13] spectrum of **1** exhibited 40 nonexchangeable protons, including nine tertiary (δ 0.99, 1.20, 1.29, 1.32, 1.42, 1.46, 1.53, 1.56, 2.01) methyl groups, and four olefinic protons (δ 5.83 [br dd, J=3.0, 5.2 Hz], 5.98 [s], 6.43, and 7.04 [each d, J=15.6 Hz]). The ^{13}C-NMR[13] spectrum of **1** displayed nine methyls, three methylenes, three methines including an oxygenated carbon (δ 71.14), six quaternary carbons including two oxygenated carbons (δ 78.03, 79.26), six olefinic carbons (δ 117.04, 120.26, 122.67, 132.79, 133.88, 152.04), and four carbonyl carbons (δ 170.29, 172.56, 202.28, 211.61). The methyl group (δ 2.01) and ester carbonyl group (δ 170.29 or 172.56) suggested the presence of an acetoxy moiety in the molecule. Seven of the 11 unsaturations were accounted for, thus implying that **1** consisted of a 4-ring system. These data showed a quite similar signal pattern and chemical shifts with those of known cucurbitacines (**3**—**6**)[14,15] except for the A ring.

The partial structure of the A ring was deduced from the heteronuclear multiple-bond correlation (HMBC, $^{2,3}J_{C-H}$= 6.3 Hz) spectrum (Fig. 1). The olefinic proton at δ 5.98 was correlated to the ester carbonyl carbon at δ 172.56, which was further correlated to two methyl groups at δ 1.32 (H_3-30) and 1.46 (H_3-29), and three quaternary carbons at δ

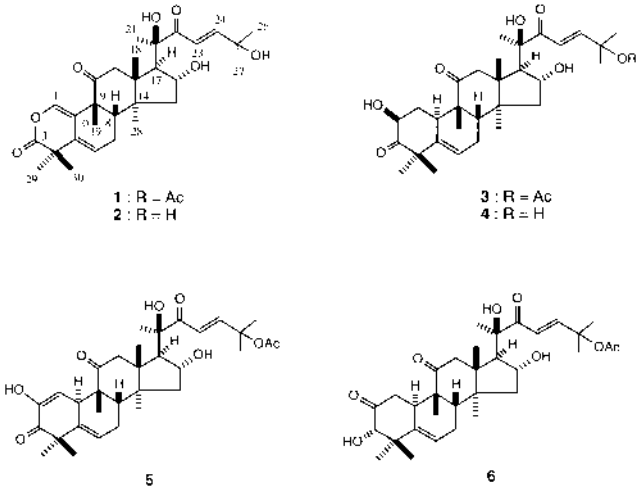

Chart 1

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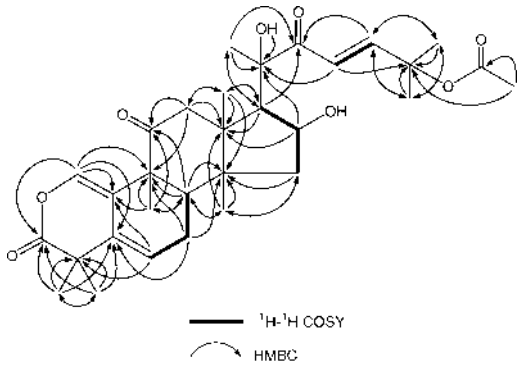

Fig. 1. ^{1}H–^{1}H and Long-Range ^{13}C–^{1}H Correlations of **1**

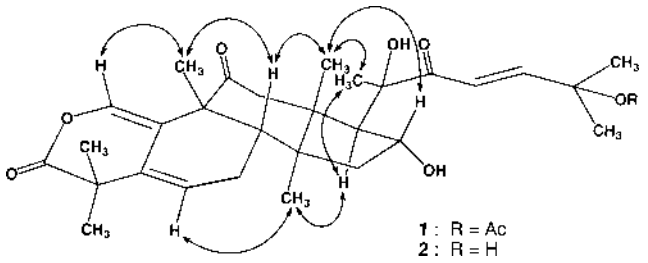

Fig. 2. NOEs of **1** and **2**

47.62 (C-9), 117.04 (C-10), and 132.79 (C-5). This indicated that **1** possesses δ-lactone on the A ring. The acetoxy group was determined to be attached at C-25 due to the four-bond correlation between the methyl group (δ 2.01) and oxygenated quaternary carbon at δ 79.26 (C-25) observed in the HMBC spectrum.[16] The other HMBC correlations, as shown by arrows in Fig. 1, allowed us to describe the planar structure of **1**.

The relative stereochemistry of **1** was identified by the nuclear Overhauser exchange spectroscopy (NOESY) spectrum, as shown in Fig. 2. The H-8 showed a cross peak to H_3-18, with further cross peaks observed to H-16 and H_3-21, and H_3-19. H-17 also showed a cross peak to H_3-21 and H_3-28. These data indicate that **1** has the same relative stereochemistry at the B—D ring and side chain as those of known cucurbitacins. Thus the structure of neocucurbitacin A was consequently established as shown in **1**.

Compound **2**, in the form of a colorless amorphous powder, $[\alpha]_D$ +82.0° (c=0.50, $CHCl_3$), gave a quasimolecular ion at m/z 499 $(M-1)^-$ in FAB-MS, and HR-FAB-MS determined the molecular formula to be $C_{29}H_{39}O_7$ ($[M-1]^-$ 499.2701, Calcd 499.2696). The IR (3441, 1761, 1696 cm^{-1}) spectrum suggested the presence of hydroxy groups and carbonyl groups. The ^{1}H-NMR[17] and ^{13}C-NMR[17] spectra of **2** showed almost the same signal patterns as those of **1** except for the absence of the acetoxy group. The upfield-shifted oxygenated quaternary carbon at δ 71.14 (C-25) strongly suggested that **2** is the deacetyl moiety of **1**. In addition, the relative stereochemistry of **2** was identified by the NOESY spectrum (Fig. 2). Thus the structure of neocucurbitacin B was determined as shown in **2**.

A large number of cucurbitacins have been isolated from the Cucurbitaceae family, although **1** and **2** are the first examples of lactone-type cucurbitacins.

Exposure of Saos-2 cells to 25 μM of **1** for 6 h resulted in a significant decrease in PEBP2αA and OCIF mRNA levels, while exposure to **2** had almost no effect on their expression. Compound **3** showed the most potent activity. The structure–activity relationships of the cucurbitacins will be reported elsewhere. Reduced expression of PEBP2αA and OCIF may lead to inhibition of bone formation and enhancement of bone resorbtion, respectively, consequently resulting in loss of bone density. We intend to identify the cellular target(s) of the cucurbitacins to understand part of the regulatory mechanism of bone remodeling.

References and Notes

1) Hakamatsuka T., Tanaka N., *Biol. Pharm. Bull.*, **20**, 464—466 (1997).
2) Komori T., Yagi H., Nomura S., Yamaguchi A., Sasaki K., Deguchi K., Shimizu Y., Bronson R. T., Gao Y.-H., Inada M., Sato M., Okamoto R., Kitamura Y., Yoshiki S., Kishimoto T., *Cell*, **89**, 755—764 (1997).
3) Otto F., Thornell A. P., Crompton T., Denzel A., Gilmour K. C., Rosewell I. R., Stamp G. W. H., Beddington R. S. P., Mundlos S., Olsen B. R., Selby P. B., Owen M. J., *Cell*, **89**, 765—771 (1997).
4) Tuda E., Goto M., Mochizuki S., Yano K., Kobayashi F., Morinaga T., Higashio K., *Biochem. Biophys. Res. Commun.*, **234**, 137—142 (1997).
5) Simonet W. S., Lacey D. L., Dunstan C. R., Kelley M., Chang M.-S., Luthy R., Nguyen H. Q., Wooden S., Bennett L., Boone T., Shimamoto G., DeRose M., Elliott R., Colombero A., Tan H.-L., Trail G., Sullivan J., Dary E., Bucay N., Renshaw-Gegg L., Hughes T. M., Hill D., Pattison W., Campbell P., Sander S., Van G., Tarpley J., Derby P., Lee R., Boyle W. J., Amgen EST Program, *Cell*, **89**, 309—319 (1997).
6) Yasuda H., Shima N., Nakagawa N., Yamaguchi K., Kinosaki M., Mochizuki S., Tomoyasu A., Yano K., Goto M., Murakami A., Tsuda E., Morinaga T., Higashio K., Udagawa N., Takahashi N., Suda T., *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 3597—3602 (1998).
7) Lacey D. L., Timms E., Tan H.-L., Kelley M. J., Dunstan C. R., Burgess T., Elliott R., Colombero A., Elliott G., Scully S., Hsu H., Sullivan J., Hawkins N., Dary E., Capparelli C., Eli A., Qian Y.-X., Kaufman S., Sarosi I., Shalhoub V., Senaldi G., Guo J., Delaney J., Boyle W. J., *Cell*, **93**, 165—176 (1998).
8) Mizuno A., Amizuka N., Irie K., Murakami A., Fujise N., Kanno T., Sato Y., Nakagawa N., Yasuda H., Mochizuki S., Gomibuchi T., Yano K., Shima N., Washida N., Tsuda E., Morinaga T., Higashio K., Ozawa H., *Biochem. Biophys. Res. Commun.*, **247**, 610—615 (1998).
9) Bucay N., Sarosi I., Dunstan C. R., Morony S., Tarpley J., Capparelli C., Scully S., Tan H. L., Xu W., Lacey D. L., Boyle W. J., Simonet W. S., *Gene. Develop.*, **12**, 1260—1268 (1998).
10) Hashimoto G. (ed.), "Illustrated Cyclopedia of Brazilian Medicinal Plants," Aboc-Sha, Yokohama, 1996, p. 388.
11) De Kock W. T., Enslin P. R., Norton K. B., Barton D. H., Sklarz B., *J. Chem. Soc.*, **1963**, 3828—3845.
12) Kupchan S. M., Groy A. H., Grove M. D., *J. Med. Chem.*, **10**, 337—340 (1967).
13) Neocucurbitacin A (**1**): Amorphous powder, HR-EI-MS m/z: 542.2877 $[M]^+$ (Calcd for $C_{31}H_{42}O_8$: 542.2880). $[\alpha]_D$ +71.3° (c=0.46, $CHCl_3$). IR (KBr) cm^{-1}: 3460, 1763, 1736, 1697. UV λ_{max} MeOH nm (log ε): 258 (3.77), 228 (4.21). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.99 (3H, s, H_3-18), 1.20 (3H, s, H_3-28), 1.28 (1H, d, J=13.4 Hz, H-15α), 1.29 (3H, s, H_3-19), 1.32 (3H, s, H_3-30), 1.42 (3H, s, H_3-21), 1.46 (3H, s, H_3-29), 1.53 (3H, s, H_3-26), 1.56 (3H, s, H_3-27), 1.89 (1H, dd, J=9.2, 13.4 Hz, H-15β), 2.01 (3H, s, $COCH_3$), 2.10 (1H, d, J=8.2 Hz, H-8), 2.20 (1H, dd, J=5.2, 20.2 Hz, H-7α), 2.47 (1H, d, J=7.0 Hz, H-17), 2.48 (1H, ddd, J=3.0, 8.2, 20.2 Hz, H-7β), 2.74 (1H, d, J=14.3 Hz, H-12β), 3.07 (1H, d, J=14.3 Hz, H-12α), 4.30 (1H, br q, J=7.0 Hz, H-16), 5.83 (1H, br dd, J=3.0, 5.2 Hz, H-6), 5.98 (1H, s, H-1), 6.43 (1H, d, J=15.6 Hz, H-23), 7.04 (1H, d, J=15.6 Hz, H-24). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 18.08 (q, C-28), 19.66 (q, C-18), 21.81 (q, C-29), 21.93 (q, $OCO\underline{CH_3}$), 23.85 (q, C-21), 24.79 (t, C-7), 24.89 (q, C-19), 25.79 (q, C-27), 26.46 (q, C-26), 28.86 (q, C-30), 41.93 (d, C-8), 42.36 (s, C-4), 44.78 (t, C-15), 47.62 (s, C-9), 48.36 (s, C-14), 50.08 (t, C-12), 50.65 (s, C-13), 58.27 (d, C-17), 71.14 (d, C-16), 78.03 (s, C-20), 79.26 (s, C-25), 117.04 (s, C-10), 120.26 (d, C-23), 122.67 (d, C-6), 132.79 (s, C-5), 133.88 (s, C-1). 152.04 (d, C-24), 170.29 (s, $O\underline{CO}CH_3$), 172.56 (s, C-3), 202.28 (s, C-22), 211.61 (s, C-11).
14) Yamada Y., Hagiwara K., Iguchi K., Takahashi Y., Suzuki S., *Chem.*

Lett., **1978**, 319—322.
15) Kupchan S. M., Meshulam H., Sneden A. T., *Phytochemistry*, **17**, 767—769 (1978).
16) The pulse-field gradient (PFG) HMBC condition: Δ=80 ms, point=1024×512, scans=128.
17) Neocucurbitacin B (**2**): Amorphous powder, negative HR-FAB-MS *m/z*: 499.2701 $[M-H]^-$ (Calcd for $C_{29}H_{39}O_7$: 499.2696). $[\alpha]_D$ +82.0° (*c*=0.50, $CHCl_3$). IR (KBr) cm^{-1}: 3441, 1761, 1696. UV λ_{max} MeOH nm (log ε): 258 (3.41), 226 (4.14). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.99 (3H, s, H_3-18), 1.19 (3H, s, H_3-28), 1.29 (3H, s, H_3-19), 1.32 (3H, s, H_3-30), 1.35 (1H, d, *J*=13.1 Hz, H-15β), 1.37 (3H, s, H_3-26), 1.38 (3H, s, H_3-27), 1.40 (3H, s, H_3-21), 1.46 (3H, s, H_3-29), 1.86 (1H, dd, *J*=9.5, 13.1 Hz, H-15β), 2.10 (1H, d, *J*=7.9 Hz, H-8), 2.18 (1H, dd, *J*=5.2, 20.2 Hz, H-7α), 2.48 (1H, ddd, *J*=3.0, 7.9, 20.2 Hz, H-7β), 2.52 (1H, d, *J*=7.0 Hz, H-17), 2.75 (1H, d, *J*=14.3 Hz, H-12β), 3.10 (1H, d, *J*=14.3 Hz, H-12α), 4.34 (1H, br t, J=7.3 Hz, H-16), 5.82 (1H, br dd, *J*=3.0, 5.2 Hz, H-6), 5.99 (1H, s, H-1), 6.64 (1H, d, *J*=15.2 Hz, H-23), 7.10 (1H, *J*=15.2 Hz, H-24). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 18.11 (q, C-28), 19.78 (q, C-18), 22.12 (q, C-29), 23.80 (q, C-21), 24.78 (t, C-7), 25.04 (q, C-19), 28.68 (q, C-30), 29.19 (q, C-26), 29.44 (q, C-27), 41.87 (d, C-8), 42.38 (s, C-4), 44.85 (t, C-15), 47.57 (s, C-9), 48.49 (s, C-14), 50.07 (t, C-12), 50.78 (s, C-13), 57.70 (d, C-17), 71.15 (d, C-16), 71.14 (s, C-25), 78.03 (s, C-20), 116.94 (s, C-10), 118.96 (d, C-23), 122.52 (d, C-6), 132.88 (s, C-5), 133.98 (s, C-1). 155.62 (d, C-24), 172.52 (s, C-3), 202.84 (s, C-22), 211.65 (s, C-11).