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Glucosylation of cinobufagin by cultured suspension cells of *Catharanthus roseus*

Min Ye, Jungui Dai, Hongzhu Guo, Yajun Cui and Dean Guo*

The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Xueyuan Road # 38, Beijing 100083, PR China

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Abstract—The biotransformation of cinobufagin by cell suspension cultures of *Catharanthus roseus* was investigated. After 6 days incubation, four new glucosylated derivatives, desacetylcinobufagin 16-*O*-β-D-glucoside, 3-*epi*-desacetylcinobufagin 16-*O*-β-D-glucoside, 3-oxo-desacetylcinobufagin 16-*O*-β-D-glucoside, and cinobufagin 3-*O*-β-D-glucoside, respectively, were isolated and identified on the basis of their physical and chemical data. This is the first report of bufadienolides with a glucosyl substituent at the C-16 position.

Bufadienolides are cardioactive C-24 steroids originally isolated from the venom of toads, such as *Bufo bufo gargarizans* Cantor, possessing A/B *cis* and C/D *cis* structures and an α-pyrone ring at the 17-position. Until now, more than 250 bufadienolides have been obtained from animal and plant sources.[1] Bufadienolides exhibit a variety of biological activities, such as cardiotonic, blood pressure stimulation, respiration and antineoplastic activities. Of these bufadienolides, resibufogenin is now used as a cardiotonic drug, and bufalin has been reported to have strong cytotoxic effects and potent differentiation-inducing activity on human myeloid leukemia cell lines (K562, U937, ML1, HL60).[2,3] Cinobufagin **1** is one of the major active constituents in toad venom (ca. 4–6% dry weight) with a characteristic 14β,15β-epoxy ring.[4,5] This functional group is common in bufadienolides from animal sources and has not, so far, been found in compounds from plants. As a closely related analogue of bufalin, cinobufagin exhibits moderate cytotoxic activities.[6] However, structural modification is still needed to produce new antineoplastic agents with enhanced activity and improved bioavailability.

Biotransformation is now becoming an increasingly important tool available to synthetic chemists in the structural modification of natural or synthetic organic compounds. In some cases, it provides potential pathways to the preparation of chemically inaccessible metabolites.[7–9] Transformation of steroids by plant cell cultures has long been investigated. A successful example was the selective hydroxylation of digitoxigenin.[10] Unfortunately, biotransformation of animal-originated bufadienolides by plant suspension cells has not been reported so far. Recently we have reported the site-specific hydroxylation of taxanes by *Ginkgo* cells.[11] In an ongoing effort to produce novel compounds of pharmaceutical interest, biotransformation of cinobufagin by a series of biological systems including plant suspension cells has been examined. We report here the glucosylation of cinobufagin by cultured suspension cells of *Catharanthus roseus* (L.) G. Don (Apocynaceae).

In the screening test, it was found that compound **1** could be effectively transformed into more polar products by cell suspension cultures of *C. roseus* (MS medium supplemented with 0.5 mg/l 6-BA, 0.5 mg/l NAA, 0.2 mg/l 2,4-D and 3% sucrose). No product was generated in the substrate control with no plant cells. For preparative transformation, 20 mg of **1** in 1 ml of EtOH was fed into a 1000 ml Erlenmeyer flask containing 400 ml of suspension cells that had been pre-cultured for 7 days. In total, 560 mg of **1** was administered. After 6 days incubation, the cells were filtered and the culture medium was extracted with EtOAc. The extract was concentrated and subjected to Si gel chromatography with $CHCl_3$/MeOH (10:1) as the eluent. Four new glucosylated products were obtained and further purified with Sephadex LH-20. On the basis of spectroscopic analysis, their structures were eluci-

Keywords: biotransformation; cinobufagin; *Catharanthus roseus*; cell suspension culture; glucosylation.

* Corresponding author. Tel.: 86-10-62091516; fax: 86-10-62092700; e-mail: gda@mail.bjmu.edu.cn

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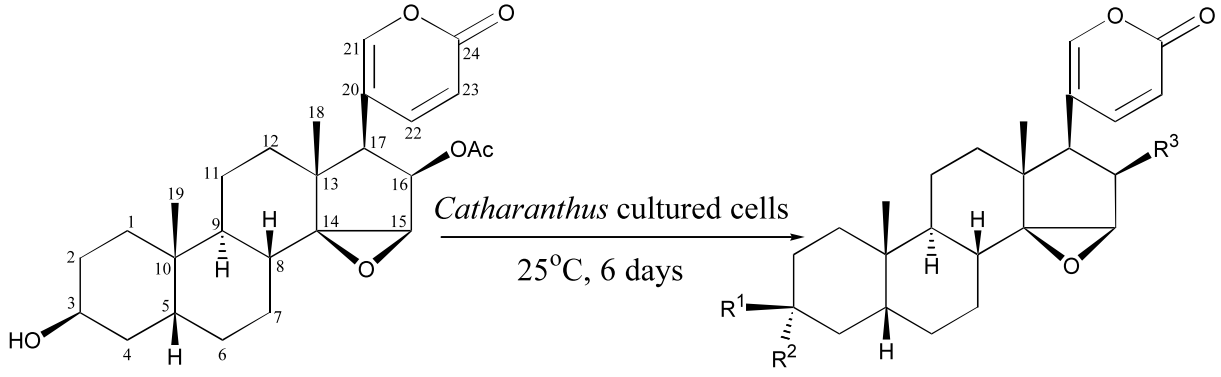
dated as desacetylcinobufagin 16-*O*-β-D-glucoside (**2**, 5.3 mg), 3-*epi*-desacetylcinobufagin 16-*O*-β-D-glucoside (**3**, 12.0 mg), 3-oxo-desacetylcinobufagin 16-*O*-β-D-glucoside (**4**, 4.7 mg), and cinobufagin 3-*O*-β-D-glucoside (**5**, 28.7 mg), respectively (Scheme 1). TOF-MS of compound **2** revealed the quasi-molecular ion at m/z 601 $[M+K]^+$ and m/z 585 $[M+Na]^+$, indicating the molecular formula of $C_{30}H_{42}O_{10}$. Detailed analysis of the ^{13}C NMR spectrum suggested that **2** was a 16-*O*-glucosylated derivative of **1**.[12] This deduction was supported by the long-range coupling between H-16 (δ 4.83) and C-1′ (δ 101.7) in the HMBC experiment, and by a NOE correlation between H-16 and H-1′(δ 3.97) in the NOESY spectrum. The coupling constant of H-1′ ($J=$ 6.0 Hz) suggested that the glucose was linked in the β-configuration. TOF-MS of **3** showed the same pseudo-molecular ion peaks as those of **2**. By comparing its ^{13}C NMR spectrum with that of **2**, it was clear that their structures were essentially similar. NOE enhancements between H-3 and H-1β, as well as between H-3 and H-5 suggested that **3** was the 3-epimerized derivative of **2**.[13,14] As another strong support, the signal for H-3 (δ 3.40) appeared as a very broad multiplet due to the couplings between axial hydrogens. The $[M+K]^+$ peak at m/z 599 and the $[M+Na]^+$ peak at m/z 583 in the TOF-MS established the molecular formula of **4** as $C_{30}H_{40}O_{10}$. The ^{13}C NMR spectrum revealed that a glucosyl residue was attached at C-16. In addition, the disappearance of the signal assigned to C-3 and the appearance of a quaternary carbon signal at δ 211.7 indicated the presence of a carbonyl group at C-3.[15] These data allowed the identification of **4** as 3-oxo-desacetylcinobufagin 16-*O*-β-D-glucoside.[16] Detailed analysis of the ^{13}C NMR spectrum suggested that **5** was the 3-glucosylated derivative of **1**.[17] This conclusion was confirmed by the long-range coupling between H-1′ (δ 4.15) and C-3 (δ 72.5) in the HMBC. In accordance, C-3 resonated at a much lower field (δ 72.5) when compared to that of cinobufagin (δ 64.5) because of glucosyl substitution. All the ^{13}C and ^1H NMR data of **2**–**5** were carefully and unambiguously assigned by extensive NMR techniques (^1H–^1H COSY, NOESY, DEPT, HMQC and HMBC).

The system pH value remained in the range of 5.50–6.00 during the transformation process. Biomass determination suggested that substrate administration did not affect the regular growth of plant cells. Time course investigation of the major product **5** was also carried out. It was indicated that the transformation rate of **5** reaches its highest level of about 15% after 72 hours incubation (determined by HPLC).

The results obtained in this study indicated that cinobufagin could be selectively glucosylated at position C-16 by cell suspension cultures of *C. roseus*. From detailed analysis of the structures of naturally occurring bufadienolides, it is apparent that bufadienolides from animal sources never occur as glycosides, but are often esterified by carboxylic acids, bicarboxylic acids, or sulphuric acid. Although bufadienolides from plant resources usually occur as glycosides, the sugars are only linked to the C-3 or less frequently, the C-5 hydroxyl groups.[1] This is the first report of bufadienolides with a sugar moiety conjugated at the hydroxyl group at C-16.

Oxirane rings have been reported to be chemically active due to their electronic polarization and the strain of the ring structure. Epoxides were also found to be easily degraded by a variety of microbial systems.[18] However, the 14β,15β-oxirane ring in cinobufagin appeared to be rather stable after several days of incubation. Therefore, transformation by plant suspension cultures can be a powerful method for the structural modification of organic compounds with chemically labile functions.

This study provides a good example of an enzyme-mediated biocatalysis reaction by cultured plant cells which exhibits the great potential and versatility of



cinobufagin **1**

2 R^1=OH R^2= H R^3=O-glc
3 R^1=H R^2=OH R^3=O-glc
4 R^1+R^2=O R^3=O-glc
5 R^1=O-glc R^2= H R^3=OAc

Scheme 1. Biotransformation of cinobufagin by *C. roseus* cell suspension cultures.

biotransformation in the structural modification of natural products.

Acknowledgements

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12. Desacetylcinobufagin 16-*O*-β-D-glucoside (**2**): white amorphous powder; $C_{30}H_{42}O_{10}$; mp 234–236°C; $[\alpha]_D^{25}$ −2.0 (*c* 0.050, MeOH); UV λ_{max} (MeOH): 207.0, 294.0 nm; IR ν_{max} (KBr): 3545, 3426, 2926, 2865, 1698, 1624, 1541, 1378, 1252, 1072, 1035 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): 7.91 (1H, *br*d, *J*=9.5, H-22), 7.48 (1H, s, H-21), 6.10 (1H, d, *J*=9.5, H-23), 4.91 (3H, *br*s, 2′, 3′, 4′-OH), 4.83 (1H, d, *J*=9.5, H-16), 4.52 (2H, *br*s, 3, 6′-OH), 3.97 (1H, *br*d, *J*=6.0, H-1′), 3.89 (1H, *br*s, H-3), 3.66 (1H, s, H-15), 3.61 (1H, m, H-6′), 3.42 (1H, m, H-6′), 3.09 (1H, m, H-5′), 3.00 (1H, m, H-3′), 2.98 (1H, m, H-4′), 2.82 (1H, *br*t, *J*=8.0, H-2′), 2.76 (1H, d, *J*=9.5, H-17), 1.90 (1H, dt, *J*=3.5, 11.5, H-8), 1.81 (1H, m, H-4), 1.76 (1H, m, H-6), 1.73 (1H, m, H-1), 1.69 (1H, m, H-5), 1.67 (1H, m, H-9), 1.64 (1H, m, H-12), 1.46 (1H, m, H-2), 1.45 (1H, m, H-11), 1.42 (1H, m, H-12), 1.39 (1H, m, H-1), 1.35 (1H, m, H-2), 1.33 (1H, m, H-7), 1.20 (1H, m, H-4), 1.17 (1H, m, H-11), 1.08 (1H, m, H-6), 0.98 (1H, m, H-7), 0.90 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): 161.4 (s, C-24), 152.2 (d, C-21), 149.4 (d, C-22), 116.0 (s, C-20), 112.5 (d, C-23), 101.7 (d, C-1′), 76.9 (d, C-5′), 76.7 (d, C-3′), 76.5 (d, C-16), 73.2 (d, C-2′), 71.5 (s, C-14), 70.0 (d, C-4′), 64.5 (d, C-3), 61.2 (t, C-6′), 61.0 (d, C-15), 48.4 (d, C-17), 44.2 (s, C-13), 38.7 (t, C-12), 38.3 (d, C-9), 35.5 (d, C-5), 35.0 (s, C-10), 32.9 (t, C-4), 32.6 (d, C-8), 29.3 (t, C-1), 27.5 (t, C-2), 25.5 (t, C-6), 23.6 (q, 19-CH_3), 20.5 (t, C-11), 20.1 (t, C-7), 16.9 (q, 18-CH_3); TOF-MS (*m*/*z*): 601.1 [*M*+K]$^+$, 585.1 [*M*+Na]$^+$, 401.2 [*M*+H-glc]$^+$. Anal. calcd for $C_{32}H_{42}O_{10}$ (%): C, 64.04; H, 7.52. Found: C, 63.81; H, 7.50.
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14. 3-*epi*-Desacetylcinobufagin 16-*O*-β-D-glucoside (**3**): colorless needles (Me_2CO); $C_{30}H_{42}O_{10}$; mp 188–189°C; $[\alpha]_D^{25}$ −4.8 (*c* 0.041, MeOH); UV λ_{max} (MeOH): 204.0, 294.0 nm; IR ν_{max} (KBr): 3409, 2931, 2866, 1707, 1625, 1538, 1450, 1374, 1251, 1078, 1036 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): 7.88 (1H, *br*s, H-22), 7.48 (1H, s, H-21), 6.10 (1H, d, *J*=9.5, H-23), 4.91 (3H, *br*s, 2′, 3′, 4′-OH), 4.84 (1H, d, *J*=8.5, H-16), 4.53 (2H, *br*s, 3, 6′-OH), 3.99 (1H, *br*d, *J*=7.5, H-1′), 3.70 (1H, s, H-15), 3.67 (1H, *br*s, H-6′), 3.42 (1H, m, H-6′), 3.40 (1H, *br*s, H-3), 3.09 (1H, *br*t, *J*=7.0, H-5′), 2.99 (1H, *br*t, *J*=8.5, H-3′), 2.96 (1H, *br*t, *J*=8.0, H-4′), 2.81 (1H, *br*t, *J*=8.0, H-2′), 2.77 (1H, d, *J*=8.5, H-17), 1.91 (1H, dt, *J*=3.0, 12.0, H-8), 1.73 (1H, m, H-6), 1.70 (1H, m, H-2), 1.68 (1H, m, H-9), 1.65 (1H, m, H-12), 1.60 (1H, *br*d, *J*=11.5, H-4), 1.52 (1H, *br*d, *J*=10.0, H-1), 1.44 (1H, m, H-11), 1.40 (1H, m, H-12), 1.34 (1H, m, H-7), 1.32 (1H, m, H-4), 1.27 (1H, m, H-5), 1.23 (1H, m, H-1), 1.19 (1H, m, H-6), 1.17 (1H, m, H-11), 1.01 (1H, m, H-7), 0.94 (1H, m, H-2), 0.88 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): 161.2 (s, C-24), 152.1 (d, C-21), 149.4 (d, C-22), 116.3 (s, C-20), 112.5 (d, C-23), 101.8 (d, C-1′), 77.0 (d, C-5′), 76.7 (d, C-3′), 76.5 (d, C-16), 73.2 (d, C-2′), 71.5 (s, C-14), 70.0 (d, C-4′), 69.7 (d, C-3), 61.2 (t, C-6′), 61.0 (d, C-15), 48.4 (d, C-17), 44.1 (s, C-13), 41.1 (d, C-5), 39.0 (d, C-9), 38.7 (t, C-12), 35.9 (t, C-4), 34.7 (t, C-2), 34.5 (s, C-10), 32.7 (d, C-8), 30.3 (t, C-1), 26.0 (t, C-6), 23.0 (q, 19-CH_3), 20.4 (t, C-11), 20.3 (t, C-7), 16.9 (q, 18-CH_3); TOF-MS (*m*/*z*): 601.1 [*M*+K]$^+$, 585.1 [*M*+Na]$^+$, 401.2 [*M*+H-glc]$^+$. Anal. calcd for $C_{30}H_{42}O_{10}$ (%): C, 64.04; H, 7.52. Found: C, 64.12; H, 7.47.
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H-6), 1.06 (1H, *br*d, $J=14.0$, H-7), 0.95 (3H, s, 19-CH_3), 0.70 (3H, s, 18-CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): 211.7 (s, C-3), 161.4 (s, C-24), 151.9 (d, C-21), 149.4 (d, C-22), 117.1 (s, C-20), 112.6 (d, C-23), 101.6 (d, C-1′), 77.0 (d, C-5′), 76.4 (d, C-3′), 76.4 (d, C-16), 73.2 (d, C-2′), 71.3 (s, C-14), 69.9 (d, C-4′), 61.1 (t, C-6′), 61.0 (d, C-15), 48.4 (d, C-17), 44.2 (s, C-13), 43.0 (d, C-5), 41.6 (t, C-4), 38.5 (d, C-9), 38.5 (t, C-12), 36.7 (t, C-2), 36.0 (t, C-1), 34.7 (s, C-10), 32.4 (d, C-8), 25.3 (t, C-6), 22.0 (q, 19-CH_3), 20.6 (t, C-11), 19.5 (t, C-7), 16.9 (q, 18-CH_3); TOF-MS (*m*/*z*): 599.1 [*M*+K]$^+$, 583.2 [*M*+Na]$^+$, 399.2 [*M*+H-glc]$^+$. Anal. calcd for $C_{30}H_{40}O_{10}$ (%): C, 64.27; H, 7.19. Found: C, 64.06; H, 7.21.

17. Cinobufagin 3-*O*-β-D-glucoside (**5**): white crystalline powder; $C_{32}H_{44}O_{11}$; mp 165–166°C; $[\alpha]_D^{25}$ −15.0 (*c* 0.033, MeOH); UV λ_{max} (MeOH): 203.0, 294.0 nm; IR ν_{max} (KBr): 3421, 2934, 1723, 1635, 1377, 1243, 1078, 1037 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): 7.87 (1H, *br*d, $J=9.5$, H-22), 7.48 (1H, s, H-21), 6.24 (1H, d, $J=10.0$, H-23), 5.48 (1H, d, $J=9.5$, H-16), 4.89 (1H, d, $J=4.5$, 3′-OH), 4.88 (1H, d, $J=4.5$, 4′-OH), 4.84 (1H, d, $J=5.0$, 2′-OH), 4.42 (1H, d, $J=6.0$, 6′-OH), 4.15 (1H, d, $J=7.0$, H-1′), 3.93 (1H, *br*s, H-3), 3.76 (1H, s, H-15), 3.64 (1H, dd, $J=5.0$, 11.0, H-6′), 3.41 (1H, m, H-6′), 3.11 (1H, m, H-3′), 3.05 (1H, m, H-5′), 3.03 (1H, m, H-4′), 2.93 (1H, m, H-2′), 2.87 (1H, d, $J=9.0$, H-17), 1.94 (1H, m, H-8), 1.82 (3H, s, 16-OAc), 1.76 (1H, m, H-6), 1.74 (1H, m, H-9), 1.72 (1H, m, H-1), 1.72 (1H, m, H-5), 1.68 (1H, m, H-12), 1.54 (1H, m, H-2), 1.51 (1H, m, H-2), 1.48 (1H, m, H-12), 1.45 (1H, m, H-4), 1.42 (1H, m, H-4), 1.33 (1H, *br*d, $J=11.0$, H-7), 1.23 (1H, m, H-1), 1.20 (1H, *br*d, $J=13.0$, H-11), 1.14 (1H, m, H-4), 1.10 (1H, *br*d, $J=13.0$, H-6), 1.00 (1H, *br*d, $J=12.5$, H-7), 0.90 (3H, s, 19-CH_3), 0.70 (3H, s, 18-CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): 169.3 (s, $CH_3\underline{C}O$), 160.8 (s, C-24), 152.2 (d, C-21), 148.5 (d, C-22), 116.1 (s, C-20), 112.9 (d, C-23), 101.0 (d, C-1′), 76.8 (d, C-3′), 76.7 (d, C-5′), 74.5 (d, C-16), 73.5 (d, C-2′), 72.5 (d, C-3), 72.0 (s, C-14), 70.1 (d, C-4′), 61.0 (t, C-6′), 59.4 (d, C-15), 49.0 (d, C-17), 44.6 (s, C-13), 38.8 (t, C-12), 38.2 (d, C-9), 35.5 (d, C-5), 34.8 (s, C-10), 32.6 (d, C-8), 29.7 (t, C-4), 29.1 (t, C-1), 26.0 (t, C-2), 25.5 (t, C-6), 23.3 (q, 19-CH_3), 20.6 (t, C-11), 20.2 (q, $\underline{C}H_3CO$), 20.0 (t, C-7), 16.9 (q, 18-CH_3); FAB-MS (*m*/*z*): 605 [*M*+H]$^+$ (25), 443 [*M*+H-glc]$^+$ (3), 185(28), 149(25), 109(32), 93(84), 55(100). Anal. calcd. for $C_{32}H_{44}O_{11}$ (%): C, 63.56; H, 7.33. Found: C, 63.38; H, 7.34.
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