# A Hepatitis B Virus Inhibitory Neolignan from *Herpetospermum caudigerum*

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**A new dihydrobenzofuran neolignan, herpepropenal, was isolated from the seeds of *Herpetospermum caudigerum*. Its chemical structure was established based on spectroscopic analysis. In this work, the inhibitory effects of herpepropenal on hepatitis B virus DNA and on the replication and expression of hepatitis B surface antigen and hepatitis B e antigen were also evaluated. The new compound exhibited inhibitory effects against hepatitis B virus.**

**Key words** *Herpetospermum caudigerum*; herpepropenal; hepatitis B virus inhibition; neolignan

The seeds of *Herpetospermum caudigerum* (Cucurbitaceae) have long been used in traditional Tibetan medicine to treat liver disease,[1)] and they have been well known to be a rich source of lignans[2—8)] with antihepatitic[8)] and hepatoprotective[1)] effects. Previous bioassay-guided separation of the ethanol extract and the ethyl acetate-soluble fraction of this plant led to the isolation of herpetin and herpetrione, which have shown inhibitory activity against the hepatitis B virus (HBV).[8)] In addition, other bioactive lignans including entisolariciresinol, dehydrodiconiferyl alcohol, herpetetrone, herpetotriol, and herpetal have also been detected in the seeds of *H. caudigerum* using RP-HPLC.[9)] As part of an ongoing investigation on the HBV-inhibitory constituents of the seeds of *H. caudigerum*, a new dihydrobenzofuran neolignan named herpepropenal (Fig. 1) that exhibits activity against HBV was discovered. Its isolation and structural identification are reported here. The inhibitory effects of herpepropenal against HBV DNA and the replication and expression of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were also evaluated.

## Results and Discussion

Herpepropenal was obtained as a yellow amorphous solid. The molecular formula $C_{30}H_{30}O_{10}$ was deduced from the high-resolution electrospray-ionization mass spectrometry (HR-ESI-MS) (*m*/*z* 573.1738; Calcd for $C_{30}H_{30}O_{10}Na^+$, 573.1737) and $^{13}$C-NMR data. The $^1$H-NMR spectrum displayed signals at $\delta_H$ 7.62 (1H, br s), 7.57 (1H, br d, $J$=7.6 Hz) and 6.74 (1H, d, $J$=7.7 Hz) for an ABX spin system of a 1,3,4-trisubstituted aromatic ring (A), and four signals at $\delta_H$ 7.22 (1H, br s), 7.20 (1H, br s), 6.84 (1H, br s), and 6.69 (1H, br s) for two tetrasubstituted aromatic rings (B and D). The remaining $^1$H-NMR signals exhibited one aldehyde proton ($\delta_H$ 9.57, 1H, d, $J$=7.5 Hz), two olefinic protons ($\delta_H$ 7.60, 1H, d, $J$=5.1 Hz; $\delta_H$ 6.64, 1H, m), and three methoxyl groups ($\delta_H$ 3.89, 3.88, 3.83, 3H each, s). The $^{13}$C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra exhibited 30 signals (Table 1), including 12 quaternary carbons, 13 methines, two oxygenated methylenes, and three methoxyls. An inspection of the $^1$H-NMR, $^{13}$C-NMR, and heteronuclear multiple bond correlation (HMBC) spectra

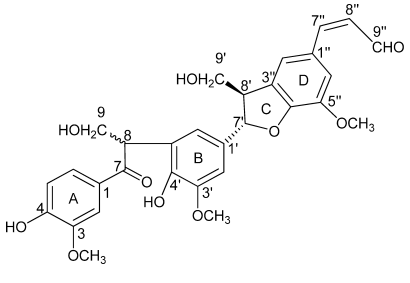


Fig. 1. Structure of Herpepropenal

Table 1. $^1$H-NMR (Mult., $J$ in Hz) at 500 MHz, $^{13}$C-NMR (Mult.) at 125 MHz and HMBC Correlations of Herpepropenal in $CD_3OD$

| Position | $\delta$ (H) | $\delta$ (C) | HMBC (H→C) |
|---|---|---|---|
| 1 | | 123.6 (s) | |
| 2 | 7.62 (br s) | 111.5 (d) | 3, 4, 6, 7 |
| 3 | | 147.6 (s) | |
| 4 | | 152.4 (s) | |
| 5 | 6.74 (d, 7.7) | 114.6 (d) | 1, 3, 4 |
| 6 | 7.57 (br d, 7.6) | 123.4 (d) | 2 |
| 7 | | 198.4 (s) | |
| 8 | 5.27 (m) | 48.2 (d) | 1, 7, 9 |
| 9 | 3.65 (m) | 63.1 (t) | |
| | 4.22 (m) | | |
| 1′ | | 132.1 (s) | |
| 2′ | 6.69 (br s) | 107.8 (d) | 3′, 4′, 6′, 7′ |
| 3′ | | 148.1 (s) | |
| 4′ | | 143.7 (s) | |
| 5′ | | 128.4 (s) | |
| 6′ | 6.84 (br s) | 117.4 (d) | 8, 2′, 4′, 5′, 7′ |
| 7′ | 5.50 (d, 6.5) | 88.8 (d) | 1′, 2′, 6′, 9′, 4″ |
| 8′ | 3.45 (m) | 53.1 (d) | 1′, 9′ |
| 9′ | 3.75 (m) | 63.2 (t) | 7′, 3″ |
| | 3.77 (m) | | |
| 1″ | | 128.6 (s) | 8″ |
| 2″ | 7.22 (br s) | 118.7 (d) | 4″, 7″ |
| 3″ | | 130.1 (s) | |
| 4″ | | 151.6 (s) | |
| 5″ | | 144.7 (s) | |
| 6″ | 7.20 (br s) | 113.3 (d) | 4″, 5″, 7″ |
| 7″ | 7.60 (d, 5.1) | 154.8 (d) | 2″, 6″, 9″ |
| 8″ | 6.64 (m) | 125.8 (d) | 1″ |
| 9″ | 9.57 (d, 7.5) | 194.8 (CHO) | 8″ |
| 3-$OCH_3$ | 3.83 (s) | 55.1 (q) | 3 |
| 3′-$OCH_3$ | 3.88 (s) | 55.3 (q) | 3′ |
| 5″-$OCH_3$ | 3.89 (s) | 55.7 (q) | 5″ |

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allowed the establishment of a dihydrobenzofuran neolignan skeleton, which was consistent with the literature.[10] In the HMBC spectrum (Fig. 2), the ketone carbonyl signal at $\delta_C$ 198.4 (C-7) correlated with the aromatic proton signals at $\delta_H$ 7.62 (H-2) and 5.27 (H-8), and the signal of H-8 further correlated with C-1, C-7, and C-9. The downfield shift of H-8 can clearly be explained by the proximity of the carbonyl and phenyl groups. The $^1$H–$^1$H correlation spectroscopy (COSY) correlations of H-8′ ($\delta_H$ 3.45, 1H, m) with the oxygenated methylene protons $H_2$-9′ ($\delta_H$ 3.74—3.78, 2H, m), and the HMBC correlations from the oxygenated methine proton H-7′ ($\delta_H$ 5.50, 1H, d, $J$=6.5 Hz) to C-9′, H-8′ ($\delta_H$ 3.45, 1H, m) to C-9′ and $H_2$-9′ to C-7′, allowed the establishment of the –O–$CH_2$–CH–CH–O– moiety. The observation of the HMBC cross-peaks of H-7′ with C-1′, C-2′, C-6′, C-9′, and C-4″, as well as H-8′ with C-1′ and C-9′, suggested that the dihydrobenzofuran moiety was connected to the aromatic ring (B). An extensive examination of the $^1$H-NMR, heteronuclear single quantum coherence (HSQC), and HMBC spectra revealed a propenal group; the partial structure was confirmed by the $^1$H–$^1$H COSY correlations of H-8″ ($\delta_H$ 6.64, 1H, m) with H-7″ ($\delta_H$ 7.60, 1H, d, $J$=5.1 Hz) and H-9″ ($\delta_H$ 9.57, 1H, d, $J$=7.5 Hz). The double bond of C-7″ ($\delta_C$ 154.8) and C-8″ ($\delta_C$ 125.8) was determined to be *cis*-disubstituted due to the small coupling constant ($J$=5.1 Hz) between H-7″ and H-8″. Both H-2″ and H-6″ showed HMBC correlations with C-7″, and HMBC cross-peaks of H-7″ with C-2″ and C-6″ were also found, which indicated that the propenal moiety was attached to C-1″. Three quaternary carbon signals at $\delta_C$ 148.1, 147.6, and 144.7 were assigned to C-3′, C-3, and C-5″, respectively, due to the HMBC correlations with the methoxyl protons.

In the nuclear overhauser effect spectroscopy (NOESY) spectrum (Fig. 3), the correlations observed between H-7′ and $H_2$-9′, H-2′ and H-8′, and H-6′ and H-8′ indicated *threo* arrangements for the phenyl and hydroxymethylene groups at C-7′ and C-8′. The relative configuration was further confirmed by comparing $^{13}$C-NMR spectral data with those of the similar compound balanophonin.[10]

The inhibitory effects of herpepropenal against HBV DNA and the replication and expression of HBsAg and HBeAg in HepG2.2.15 cells were evaluated in this study. The HepG2.2.15 cell line, derived through transfection of a recombinant plasmid of two HBV DNAs with head-to-tail tandem viral genomes, could asexually replicate *in vitro*, stably secrete HBsAg and HBeAg, and produce Dane particles and replicative intermediates in the long term.[11,12] Thus it was considered a good model for screening anti-HBV drugs *in vitro*. In our experiment, the cytotoxicity of herpepropenal was measured in cultured HepG2.2.15 cells with the inhibitor of HBV reverse transcriptase [lamivudine (3TC)] as the positive control. As shown in Table 2, the values of the selection index (SI) used for evaluating the potential clinical applications of the drug exceeded 2, indicating that herpepropenal had marked inhibitory effects against HBsAg, HBeAg, and HBV DNA in the HepG2.2.15 cell line with low toxicity. The effects of different concentrations of herpepropenal and 3TC on the secretion of HBsAg and HBeAg were investigated. As shown in Table 3, there was an obvious dose-dependent effect against the replication and expression of HBsAg and HBeAg within the concentration range of 25—200 $\mu$g/ml; however, little change was found at concentrations greater than 200 $\mu$g/ml and as the amount of herpepropenal increased. The inhibitory effects of herpepropenal (25—200 $\mu$g/ml) on HBV DNA was also assessed; treatment of HepG2.2.15 cells with herpepropenal and 3TC at various concentrations (25—200 $\mu$g/ml) for 8 d resulted in a good linear correlation (Table 3). The IR values of herpepropenal which inhibited the replication of HBV DNA were 14.31%, 26.12%, 35.39%, and 42.34%, respectively, at 25, 50, 100, and 200 $\mu$g/ml. The positive control, the 3TC-treated group, inhibited HBV DNA replication at rates of 12.82%, 13.17%, 35.18%, and 40.12% under the same experimental conditions. The results show that herpepropenal is effective in reducing the replication of HBV DNA.

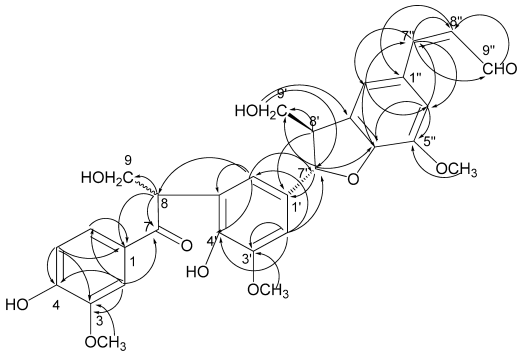


Fig. 2. Key HMBC Correlations of Herpepropenal

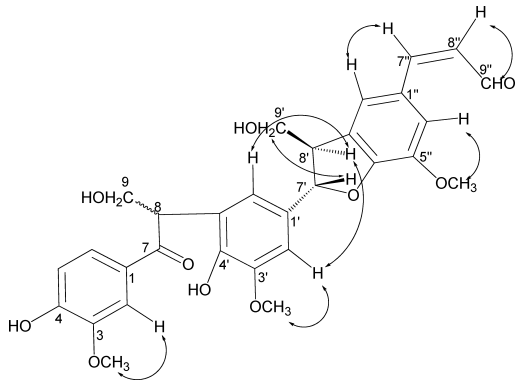


Fig. 3. Key NOESY Correlations of Herpepropenal

Table 2. Selective Inhibitory Effects of Herpepropenal on HBsAg, HBeAg and HBV DNA

| Group | $TC_{50}$ ($\mu$g/ml) | HBsAg | | HBeAg | | HBV DNA | |
|---|---|---|---|---|---|---|---|
| | | $IC_{50}$ ($\mu$g/ml) | SI | $IC_{50}$ ($\mu$g/ml) | SI | $IC_{50}$ ($\mu$g/ml) | SI |
| Herpepropenal | 401.45±23.14 | 156.51±18.91 | 2.57 | 139.67±17.05 | 2.87 | 295.02±4.24 | 2.12 |
| 3TC | 1071.01±51.12 | >400 | | >400 | | 240.30±74.93 | 4.46 |

Selection index (SI)=$TC_{50}/IC_{50}$.

Table 3. Inhibitory Effects of Herpepropenal on HBsAg, HBeAg and HBV DNA

| Dose ($\mu$g/ml) | HBsAg inhibitory rate (IR) (%) | | HBeAg inhibitory rate (IR) (%) | | HBV DNA inhibitory rate (IR) (%) | |
|---|---|---|---|---|---|---|
| | Herpepropenal | 3TC | Herpepropenal | 3TC | Herpepropenal | 3TC |
| 25 | 20.13 | 1.29 | 16.38 | 0.00 | 14.31 | 12.82 |
| 50 | 31.24 | 2.26 | 35.25 | 1.18 | 26.12 | 13.17 |
| 100 | 42.32 | 7.14 | 53.26 | 4.26 | 35.39 | 35.18 |
| 200 | 51.25 | 9.32 | 57.19 | 7.63 | 42.34 | 40.12 |

P/N=(cpm in test group−cpm in blank control group)/(cpm in positive control group−cpm in blank control group). HBsAg and HBeAg (IR)=[control (P/N)−experiment (P/N)]/[control (P/N)−2.1]×100%. HBV DNA(IR)=[OD (before treatment)−OD (after treatment)]/OD (before treatment)×100%.

In summary, the results of our study demonstrate that herpepropenal is effective in inhibiting the secretion of HBV antigens from virus-infected HepG2.2.15 cells in a dose-dependent manner and has a favorable cytotoxicity profile. Taken together, the data warrant the further preclinical evaluation of herpepropenal in hepadnavirus infections, indicating that herpepropenal might be a candidate for the development of anti-HBV agents.

## Experimental

**General Experimental Procedures** Melting point was measured on a SGW X-4 melting point apparatus and was uncorrected. Optical rotation was determined on a JASCO P-1030 polarimeter. HR-ESI-MS spectrum was acquired using a Q-Tof micro YA019 mass spectrometer. NMR experiments were performed on a Bruker AVANCE-500 spectrometer. HPLC purifications were carried out on a Waters 1525/2998 liquid chromatograph. Column chromatography (CC) was performed on Sephadex LH-20 (Pharmacia) and YMC ODS-A (50 $\mu$m) columns. Vacuum liquid chromatography (VLC) was performed on silica gel (200—300 mesh, Yantai, P. R. China). Fractions were monitored with TLC (HSGF 254, Yantai, P. R. China), and spots were visualized by heating silica gel plates sprayed with 10% $H_2SO_4$ in $H_2O$.

**Plant Material** The seeds of *H. caudigerum* were collected from Tibet, P. R. China, in 2007 and identified by Prof. Hai-Long Yuan (302 Hospital of the People's Liberation Army (PLA) and PLA Institute of Chinese Materia Medica, P. R. China). A voucher sample (NO. 0706-2) was deposited in the Department of Pharmacy, Changzheng Hospital, Second Military Medical University, P. R. China.

**Extraction and Isolation** The dried seeds of *H. caudigerum* (5 kg) were extracted with 95% ethanol (EtOH) (3×25 l) at 85 °C and were then successively partitioned with petroleum ether (3×1000 ml) and ethyl acetate (EtOAc) (3×1000 ml). The EtOAc-soluble extract (51.4 g) was subjected to VLC on silica gel using $CH_2Cl_2$–$CH_3OH$ (30 : 1, 25 : 1, 10 : 1, 5 : 1, 2 : 1, v/v) as eluent to produce three fractions (A—C). Fraction A (14.5 g) was subjected to RP-18 column chromatography ($CH_3OH$–$H_2O$ 3 : 7) and was further purified by HPLC (YMC-Pack ODS-A C18, 5 $\mu$m, 20×250 mm, 3 ml/min, UV detection 342 nm, $t_R$=57 min) eluting with $CH_3OH$–$H_2O$ (50 : 50) to yield herpepropenal (3.4 mg).

Herpepropenal, (*Z*)-3-(2-(4-hydroxy-3-(3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-oxopropan-2-yl)-5-methoxyphenyl)-3-(hydroxymethyl)-7-methoxy-2,3-dihydrobenzofuran-5-yl)acrylaldehyde: yellow amorphous solid ($CH_3OH$); mp 119—120 °C; $[\alpha]_D^{26}$ −30.3° (*c*=1.00, $CH_3OH$); HR-ESI-MS (positive-ionization mode) *m/z*: 573.1738 $[M+Na]^+$ (Calcd for $C_{30}H_{30}O_{10}Na^+$, 573.1737); $^1$H- and $^{13}$C-NMR: see Table 1.

**Activity Assay** Cell Culture: The human HBV-infected cell line HepG2.2.15 was provided by the Biotechnology Research Institute of Chinese Academy of Medical Sciences. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma), 380 $\mu$g/ml G418 (Sigma), and penicillin–streptomycin 210 U/ml (Gibco) at 37 °C in an atmosphere of 5% $CO_2$. The culture medium was changed with fresh substratum every 2 d.

**Toxicity Measurements** Toxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, with survival rates measured at the end of each experiment. 3TC was used as the positive control. Cells were seeded in 96-well tissue culture plates at a density of $2\times10^5$ cells/well for 48 h, and the supernatant was then aspirated. Herpepropenal and 3TC of different concentrations were added to the HepG2.2.15 cell media. The cells were incubated for an additional 48 h. MTT solution (10 ml/100 ml medium) was then added to each well of the assay plate, and incubation continued at 37 °C for 4 h. Dimethyl sulfoxide (DMSO) was added until it was completely dissolved, then the plates' lysates were read directly with a microplate reader at a wavelength of 595 nm. Each experiment was performed in triplicate.

**HBsAg and HBeAg Assay** The HBsAg and HBeAg assay was performed as described previously[8] with 3TC as the positive control.

**HBV DNA Assay** The HBV DNA assay was carried out according to the method described previously,[8] with 3TC as the positive control.

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## References

1) Zhang M., Deng Y., Zhang H. B., Su X. L., Chen H. L., Yu T., Guo P., *Chem. Pharm. Bull.*, **56**, 192—193 (2008).
2) Kaouadji M., Favre-Bonvin J., Mariotte A.-M., *Phytochemistry*, **17**, 2134—2135 (1978).
3) Kaouadji M., Favre-Bonvin J., Mariotte A.-M., *Bioscience*, **34C**, 1129—1132 (1979).
4) Kaouadji M., Favre-Bonvin J., *Tetrahedron Lett.*, **24**, 5881—5884 (1983).
5) Kaouadji M., Pieraccini E., *Tetrahedron Lett.*, **25**, 5135—5136 (1984).
6) Kaouadji M., Favre-Bonvin J., *Tetrahedron Lett.*, **25**, 5137—5138 (1984).
7) Kaouadji M., Favre-Bonvin J., Sarrazin F., Davoust D., *J. Nat. Prod.*, **50**, 1089—1094 (1987).
8) Yuan H. L., Yang M., Li X. Y., You R. H., Liu Y., Zhu J., Xie H., Xiao X. H., *Chem. Pharm. Bull.*, **54**, 1592—1594 (2006).
9) Cong L. B., Wang Q., Huang Y. J., Yuan H. L., Gong Q. F., Yang M., Xiao X. H., *Chin. Pharm. J.*, **43**, 946—949 (2008).
10) Haruna M., Koube T., Ito K., Murata H., *Chem. Pharm. Bull.*, **30**, 1525—1527 (1982).
11) Sells M. A., Zelent A. Z., Shvartsman M., Acs G., *J. Virol.*, **62**, 2836—2844 (1988).
12) Korba B. E., Gerin J. L., *Antiviral Res.*, **19**, 55—70 (1992).