

Identification of modification sites on human serum albumin and human hemoglobin adducts with houttuynin using liquid chromatography coupled with mass spectrometry

Zhipeng Deng, Dafang Zhong and Xiaoyan Chen*

ABSTRACT: Houttuynin, a β -keto aldehyde compound, is the major active ingredient in herba houttuyniae injection. The injection was once used as an anti-inflammatory drug associated with occasional serious hypersensitivity reactions in the clinic, which were proposed to be related to the formation of protein adducts. N^{α} -Boc-lysine, FEEM and IVTNTT were used as model amino acids or peptides containing one nucleophilic residue to investigate adduct types by liquid chromatography coupled with ion trap mass spectrometry (LC/MSⁿ) and high-resolution quadrupole time-of-flight mass spectrometry (Q-TOF MS). These adducts were respectively characterized as Schiff bases formed by 1:1 reaction of houttuynin with lysine or N -terminal residue and pyridinium adducts by 2:1 reaction. LC/MSⁿ analysis of trypsin digests of HSA/Hb incubations with houttuynin revealed that houttuynin-modified HSA adducts were formed mainly at N -terminal amino acid and lysine residues, specifically at Lys-212, Lys-414 and Lys-525 for Schiff base adducts, and at Lys-414 and Lys-432 for pyridinium adducts, and houttuynin adducted more readily with N -terminal valine of the α - and β -chains in Hb and lysine amine (Lys-62) of the β -chain for Schiff base adducts. The results showed the direct modification of houttuynin to HSA/Hb *in vitro*, which was speculated to be responsible for the adverse reactions induced by houttuyniae injection. Copyright © 2012 John Wiley & Sons, Ltd.

Supporting information can be found in the online version of this article.

Keywords: houttuynin; human serum albumin; human hemoglobin; LC/MSⁿ; adduct types; modification sites

Introduction

Recently more and more data collected from *in vitro* and *in vivo* studies have suggested an important and causative role for protein adducts in the toxicity of xenobiotics/drugs (Zhou, 2003; Evans *et al.*, 2004; Antunes *et al.*, 2010; Boerma *et al.*, 2011). Houttuynin (decanoyl acetaldehyde) is a major active ingredient in herba houttuyniae injections (Lai *et al.*, 2005; Liu *et al.*, 2009; Yuan *et al.*, 2007), which were once widely used in the clinic as an anti-inflammatory treatment. Serious and occasionally fatal allergic reactions have been reported in patients receiving therapy with the injections (Qing *et al.*, 2006; Wei *et al.*, 2004). Houttuynin has a β -keto aldehyde unit as an important pharmacological moiety. Aldehydes or aldehyde-like intermediates, such as formaldehyde, acetaldehyde, acrolein and 4-hydroxy-2-nonenal, have been reported to react with peptides/proteins to form aldehyde-derived adducts, which are responsible for drug-induced immunotoxic effects (Gubisne-Haberle *et al.*, 2004; Khan *et al.*, 2002; Israel *et al.*, 1992; Niemelä, 2001; Doorn and Petersen, 2002; Fenaille *et al.*, 2003; Roller *et al.*, 2002).

Human serum albumin (HSA) is the most abundant protein in blood, and it contains numerous nucleophilic residues, which are common targets of reactive species of drugs. *In vivo* rat tissue distribution experiments have shown that houttuynin is capable of covalently binding to plasma protein and

hemoglobin (Hb) in erythrocytes after an intravenous administration of [$1\text{-}^{14}\text{C}$] sodium houttuynfonate (Deng *et al.*, 2012).

The aim of this study was to identify the structures of houttuynin-modified amino acid and peptides, and further investigate the modification sites and structures of adducts generated in the houttuynin-modified HSA and Hb by liquid chromatography coupled with ion trap mass spectrometry (LC/MSⁿ). Sodium houttuynfonate, an adduct of houttuynin with sodium bisulfite, was used to perform the study owing to its good water solubility (Deng *et al.*, 2012).

Experimental

Materials and reagents

Sodium houttuynfonate (98.9% purity) was obtained from Qingping Pharmaceutical Co. Ltd (Shanghai, China). Houttuynin dimer was

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Abbreviations used: Hb, hemoglobin; HSA, human serum albumin.

synthesized in the laboratory and was identified by MS and NMR data (see Supporting Information). HSA (Fraction V) and Hb were purchased from Sigma-Aldrich (St Louis, MO, USA). *N*^ε-Boc-lysine, FEEM and IVTNTT were purchased from Shanghai GL Biochem Co. Ltd (Shanghai, China). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (St Louis, MO, USA). HPLC-grade formic acid (FA) and trifluoroacetic acid (TFA) were from Tedia (Fairfield, OH, USA). Other chemicals were of analytical-reagent grade. Purified water obtained by means of a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the experiment.

Houttuynin or houttuynin dimer incubation with *N*^ε-Boc-lysine and peptides

N^ε-Boc-Lysine (1.0 mM) and peptides (FEEM, IVTNTT; each 1.0 mM) were incubated with sodium houttuynifonate (1.0 mM) in 200 μ L of 100 mM phosphate buffered saline (PBS, pH=7.4) at 37°C for 60 min. The resulting samples were used to identify the adduct types by LC/MSⁿ analysis.

N^ε-Boc-lysine (1.0 mM) was incubated with sodium houttuynifonate (1.0 mM) or houttuynin dimer (1.0 mM) in 100 mM PBS (200 μ L, pH 7.4) at 37°C. Reactions were terminated at predetermined time points (1, 3, 10, 30 and 60 min), and the obtained samples were immediately subjected to LC/MSⁿ analysis.

Houttuynin incubation with HSA/Hb and trypsin digestion

HSA (0.25 mM) and Hb (0.25 mM) were incubated with sodium houttuynifonate (1.0 mM, 1:4 molar ratio) in 100 mM PBS (200 μ L, pH 7.4) at 37°C. After 60 min, the incubation was quenched with ice-cold acetonitrile at a 1:3 volume ratio. A half-volume of the incubation samples was treated by the addition of NaBH₄ (10 mM). NaBH₄ was allowed to reduce the samples for 2 h at room temperature. The reduced and remaining (nonreduced) HSA/Hb samples were dialyzed through a Microcon YM-10 centrifugal filtration device (Millipore, Bedford, MD, USA) to remove the buffer and other compounds with a molecular weight of <10,000 Da. The resulting adducted protein was dissolved in 50 mM ammonium bicarbonate/6 M guanidine hydrochloride (pH=8.3), and disulfides were reduced with dithiothreitol (30 μ Mol) for 1 h at 60°C. The alkylation of cysteine thiols with iodoacetamide (60 μ Mol) for 1 h at room temperature in the dark followed. Reaction mixtures were then centrifuged using a Microcon YM-10 system to remove the reactants with a molecular weight of <10,000 Da. The houttuynin-modified HSA and Hb samples were reconstituted in 100 mM ammonium bicarbonate solution (pH=7.8) and digested with 2% (w/w) trypsin overnight at 37°C.

Sample preparation

All the incubation samples except for the incubations containing houttuynin dimer and *N*^ε-Boc-lysine were loaded onto an Oasis[®] HLB SPE C₁₈ cartridge (30 mg, 1 mL; Waters, Milford, MA, USA). SPE cartridges were pretreated sequentially with 2 $\times$ 1 mL of methanol and 2 $\times$ 1 mL of water. After sample loading, cartridges were washed with 1 mL of water, then the houttuynin-modified amino acids/peptides were eluted with 1 mL of methanol. The eluate was evaporated to dryness at 30°C under a gentle stream of N₂ in a TurboVap evaporator (Zymark, Hopkinton, MA, USA), and the residue was reconstituted in 200 μ L of CH₃CN–H₂O (50:50, v/v). A 20 μ L of aliquot was injected for LC/MSⁿ analysis.

Instrumentation and analytical methods

LC/MSⁿ analysis for all the incubation samples was performed on an Agilent 1200 HPLC system coupled with a 6330 MSD Trap XCT Ultra

(Agilent Technologies, Waldbronn, Germany). The mass spectrometer was equipped with an ESI source in the positive-ion detection mode. The MS detector settings were set at the following values: nebulizer pressure, 40 psi (N₂); dry gas, 12 L/min (N₂); dry gas temperature, 350°C (N₂); spray capillary voltage, 3500 V; skimmer voltage, 40 V; scan range, *m/z* 200–2000; fragmentation amplitude, 0.8–1.0 V.

The LC/MSⁿ analysis of the incubation samples containing houttuynin and *N*^ε-Boc-lysine was performed with a Zorbax SB-C₁₈ column (150 $\times$ 4.6 mm, 5 μ m; Agilent, Minnesota, USA) protected by a Security-Guard C₁₈ column. The mobile phase was a mixture of methanol–water (95:5, v/v) running at a constant flow-rate of 0.6 mL/min, and the UV detector was set at 260 nm. The total run time was 6.7 min, and the injection volume was 20 μ L. Quantification was performed using multiple reaction monitoring (MRM) of the transitions *m/z* 427.4 $\rightarrow$ 327.3 for Schiff base adduct and *m/z* 589.5 $\rightarrow$ 360.4 for pyridinium adduct, respectively.

The LC/MSⁿ analysis of the incubation samples containing houttuynin and peptides were performed with a Venusil MP C₁₈ column (50 $\times$ 4.6 mm, 5 μ m; Agela, Newark, DE, USA) protected by a Security-Guard C₁₈ column. The mobile phase was acetonitrile with 0.1% formic acid (solvent A) and water with 0.1% formic acid (solvent B) running at a constant flow-rate of 0.4 mL/min. The solvent gradient was initially held at 20% A for 0.5 min and then increased linearly to 80% A over another 0.5 min, then kept at 80% A for the next 3.5 min, then finally decreased to 20% A in 0.5 min, where it was held constant at 20% A for 3 min before the next sample injection. The total run time was 8.0 min, and the injection volume was 20 μ L.

Chromatographic separation of the tryptic digested houttuynin–HSA and houttuynin–Hb conjugates was obtained through a Venusil MP C₁₈ column (50 $\times$ 4.6 mm, 5 μ m; Agela, Newark, DE, USA) protected by a Security-Guard C₁₈ column. The mobile phase was a gradient of a mixture of acetonitrile containing 0.1% FA and 0.02% TFA (solvent A) and 0.1% aqueous formic acid (solvent B). An initial 20% A was maintained for 3 min, and then increased linearly to 60% A in 7 min. It was finally decreased to 20% A in 0.5 min, where it was maintained at 20% A for 5 min. The flow-rate was 0.6 mL/min and the injection volume was 20 μ L. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 2.5 min after sample injection.

High-resolution MS analysis was performed on a quadrupole time-of-flight hybrid mass spectrometer (Synapt Q-TOF MS, Waters, Manchester, UK) employing an electrospray ionization (ESI) source. The MS detector settings were set at the following values: resolution, 10,000 (FWHM); capillary voltage, 2.7 kV; cone voltage 20 V; nebulization gas, 700 L/h (N₂); desolvation temperature, 300°C; source temperature, 120°C.

Results and discussion

Identification of two types of houttuynin–*N*^ε-Boc-lysine adducts

Peaks of two amino acid adducts (M1 and M2) were observed at 3.7 and 5.3 min, respectively (Fig. 1). High-resolution MS analysis revealed that M1 contained two nitrogen atoms (C₂₃H₄₃N₂O₅, Table 1) and exhibited a prominent protonated molecule [M+H]⁺ at *m/z* 427.4, 180 Da higher than that of the *N*^ε-Boc-lysine standard, indicating that the addition of one molecule of houttuynin to *N*^ε-Boc-lysine was accompanied by the loss of one water molecule. In the MS/MS spectrum of M1 (Fig. 2a), two diagnostic fragment ions at *m/z* 371.2 and 327.3 were also 180 Da higher than those in the MS/MS of *N*^ε-Boc-lysine, corresponding to a neutral loss of butyl (–56 Da) and butoxycarbonyl (–Boc, –100 Da), respectively. The adduct could be easily reduced by NaBH₄,

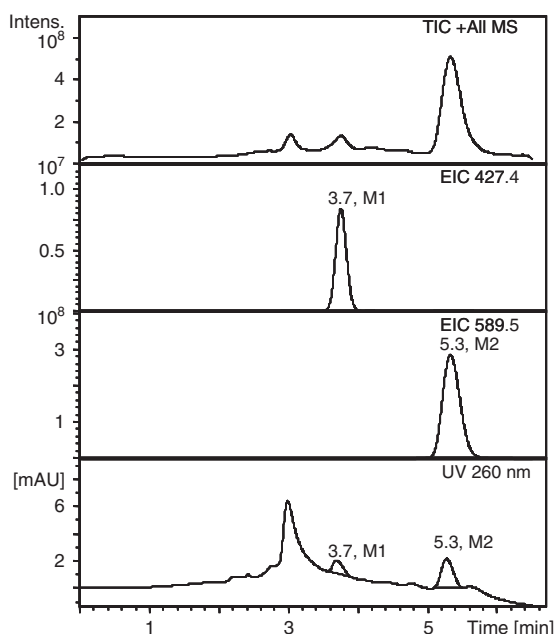


Figure 1. LC/MSⁿ analysis of the incubation samples of *N*^ε-Boc-lysine with houttuynin, and the ultraviolet spectra (260 nm) of the incubation samples.

resulting in a novel and stable adduct with an expected mass increment of 4 Da produced by the elimination of hydrogen. Based on the data, M1 was proposed to be a houttuynin-*N*^ε-Boc-lysine Schiff base.

To further identify the adduct structure, a reduction product (M3) of the Schiff base adduct was synthesized and purified. The obtained authentic compound was characterized by MS and NMR spectra. Based on the NMR data of M3, we concluded that the aldehyde group of houttuynin, not its ketone group, reacted with the primary amino group of *N*^ε-Boc-lysine to form a Schiff base adduct (see Supporting Information).

High-resolution MS analysis revealed that M2 displayed a strong molecular ion *M*⁺ at *m/z* 589.5 (C₃₅H₆₁N₂O₅, Table 1), 342 Da higher than that of *N*^ε-Boc-lysine standard, indicating the addition of two molecules of houttuynin to *N*^ε-Boc-lysine with a loss of three water molecules. The MS/MS spectrum of M2 gave the fragment ions at *m/z* 533.4 (C₃₁H₅₃N₂O₅; -butyl, -56 Da), *m/z* 489.4 (C₃₀H₅₃N₂O₃; -Boc, -100 Da) and *m/z* 360.4 (C₂₄H₄₂NO; -229 Da). M2 was prepared and assigned as a

houttuynin-*N*^ε-Boc-lysine pyridinium adduct according to its MSⁿ and NMR data (see Supporting Information).

To investigate the formation mechanisms of the Schiff base and pyridinium adducts, incubation experiments of *N*^ε-Boc-lysine with houttuynin and houttuynin dimer were carried out. The relative MS intensities of the two types of adducts derived from houttuynin and houttuynin dimer at different incubation times are shown in Fig. 3. The results showed that Schiff base adducts formed more rapidly than pyridinium adducts in a houttuynin-supplemented system. Minor Schiff base adducts (*m/z* 427) were observed in the dimer-containing system, indicating that the houttuynin dimer could be degraded into a monomer in aqueous solution. Pyridinium adducts were formed principally in the houttuynin-containing system, not in the dimer-containing system. Therefore, a plausible formation pathway for both adducts is proposed in Fig. 4. First, the lysine primary amino group or *N*-terminal α-NH₂ group of proteins attacks the aldehyde carbon of houttuynin to induce the formation of an imine derivative (I) via a Schiff base mechanism. The derivative then undergoes aldol condensation reaction with another houttuynin molecule to generate the conjugated compound (II). Then compound II is transferred to compound III by intramolecular dehydration. Compound III undergoes intramolecular rearrangement to generate the conjugated compound IV, subsequently its secondary amino group reacts with the keto carbon to produce a stable pyridinium derivative (V) by intramolecular cyclization and dehydration.

Identification of houttuynin-peptide adducts

In the incubation system containing houttuynin and peptide FEEM, two chromatographic peaks (P1 and P2, *t*_R = 3.6 min) were observed, corresponding to ions at *m/z* 735.0 and *m/z* 897.2 (Fig. 5a). The protonated molecule [*M* + H]⁺ at *m/z* 735 was 180 Da higher than that of the peptide FEEM standard. This indicated that the addition of one molecule of houttuynin to peptide FEEM was accompanied by the loss of one water molecule. In the MS/MS spectrum (Fig. 6a), major diagnostic fragment ions, such as *b*₂⁺ (*m/z* 457.0) and *b*₃⁺ (*m/z* 586.5), were also 180 Da higher than those in the MS/MS spectrum of peptide FEEM, indicating that houttuynin adducted at *N*-terminal phenylalanine and the resulting adduct was the Schiff base.

P2 exhibited the molecular ion *M*⁺ at *m/z* 897.2, which was 342 Da higher than that of peptide FEEM standard, indicating

Table 1. High-resolution mass spectral data of the schiff base adduct (*m/z* 427.4) and pyridinium adduct (*m/z* 589.5) acquired by Waters Q-TOF Synapt mass spectrometer

Adduct	Measured	Calculated	Formula	Error	DBE	
	mass	mass		mDa	PPM	
<i>m/z</i> 427.4	427.3170	427.3172	C ₂₃ H ₄₃ N ₂ O ₅	−0.2	−0.47	3.5
MS/MS	371.2532	371.2546	C ₁₉ H ₃₅ N ₂ O ₅	−1.4	−3.8	3.5
	327.2633	327.2648	C ₁₈ H ₃₅ N ₂ O ₃	−1.5	−4.6	2.5
<i>m/z</i> 589.5	589.4554	589.4575	C ₃₅ H ₆₁ N ₂ O ₅	−2.1	−3.6	6.5
MS/MS	533.3930	533.3949	C ₃₁ H ₅₃ N ₂ O ₅	−1.9	−3.6	6.5
	489.4033	489.4051	C ₃₀ H ₅₃ N ₂ O ₃	−1.8	−3.6	5.5
	360.3248	360.3261	C ₂₄ H ₄₂ NO	−1.3	−3.7	4.5

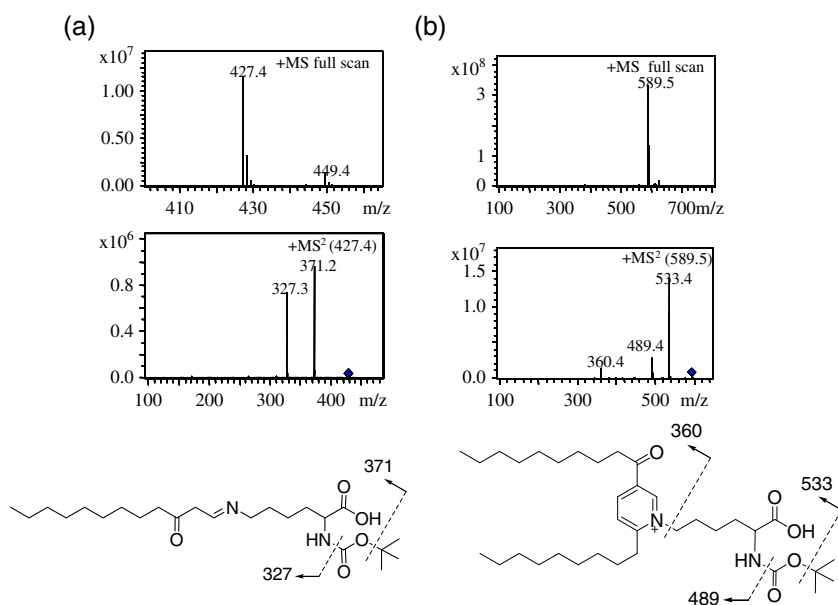


Figure 2. MS and MS/MS spectra of houttuynin-modified N^ϵ -Boc-lysine. (a) Schiff base adduct (M1, m/z 427.4); (b) pyridinium adduct (M2, m/z 589.5).

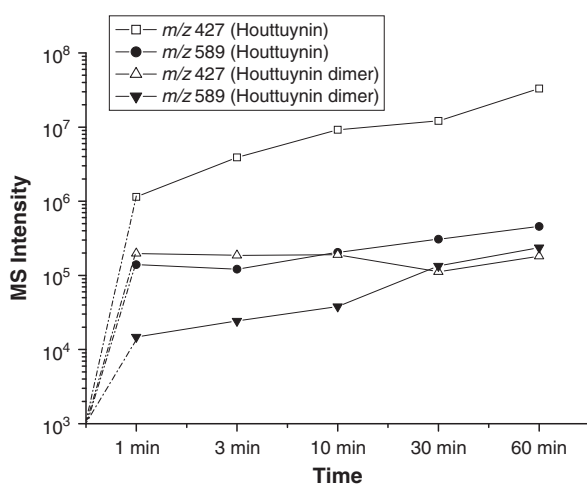


Figure 3. Reaction of N^ϵ -Boc-lysine with houttuynin and its dimer. N^ϵ -Boc-lysine (1.0 mM) was incubated with equimolar concentration of houttuynin or its dimer (1.0 mM) in 100 mM PBS.

the addition of two molecules of houttuynin to peptide FEEM with the loss of three water molecules (Fig. 5a). The MS/MS spectrum gave the fragment ions b_1^* (m/z 490.1) and a_2^* (m/z 591.3), indicating that houttuynin adducted at N -terminal phenylalanine and that the resulting adduct was the pyridinium adduct (Fig. 6b).

In the incubation system containing houttuynin and peptide IVTNTT, two chromatographic peaks (P3, m/z 828.2, $t_R = 3.4$; P4, m/z 990.38, $t_R = 3.6$ min) were observed (Fig. 5b). The protonated molecule $[M + H]^+$ at m/z 828.2 was 180 Da higher than that of the peptide IVTNTT standard. This indicated that the addition of one molecule of houttuynin to peptide IVTNTT was accompanied by the loss of one water molecule. In the MS/MS spectrum (Fig. 7a), $[M + H - NH_3]^+$ (m/z 811.1), $[M + H - H_2O]^+$ (m/z 810.1), b_5^* (m/z 709.5) and $y_5 - H_2O$ (m/z 418.0) were observed. The MS³ spectrum of the m/z 810.1 ($828.2 \rightarrow 810.1$) showed fragment ions of m/z 792.1 (-18 Da, $-H_2O$), m/z 774.1 (-36 Da, $-2H_2O$), m/z 639.9 (-170 Da, $-C_{11}H_{22}O$), m/z 417.9 (-392 Da, $y_5 - H_2O$), m/z 316.8 (-493 Da, $y_3 - H_2O$). The above data indicated that P3 was Schiff base.

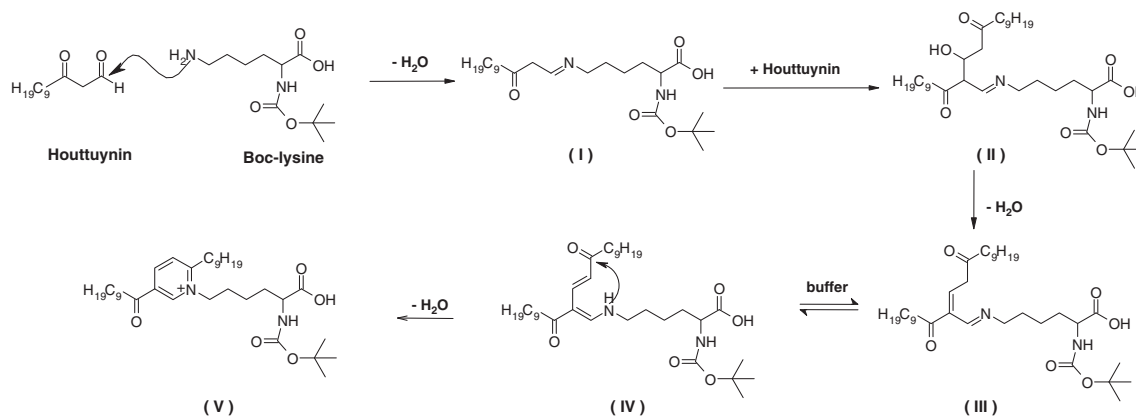


Figure 4. Proposed mechanisms for formation of houttuynin-lysine adduct.

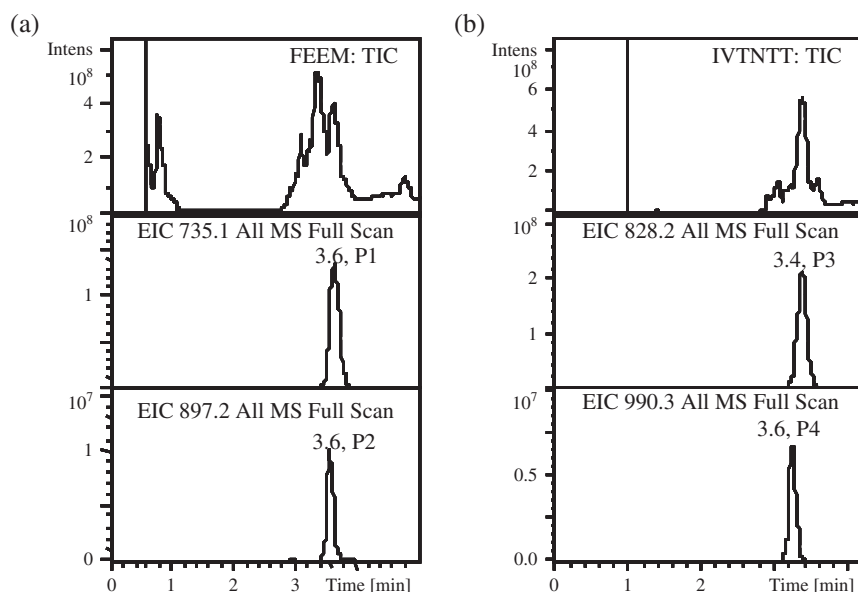


Figure 5. LC/MSⁿ analysis of houttuynin-modified peptides. (a) FEEM; (b) IVTNTT.

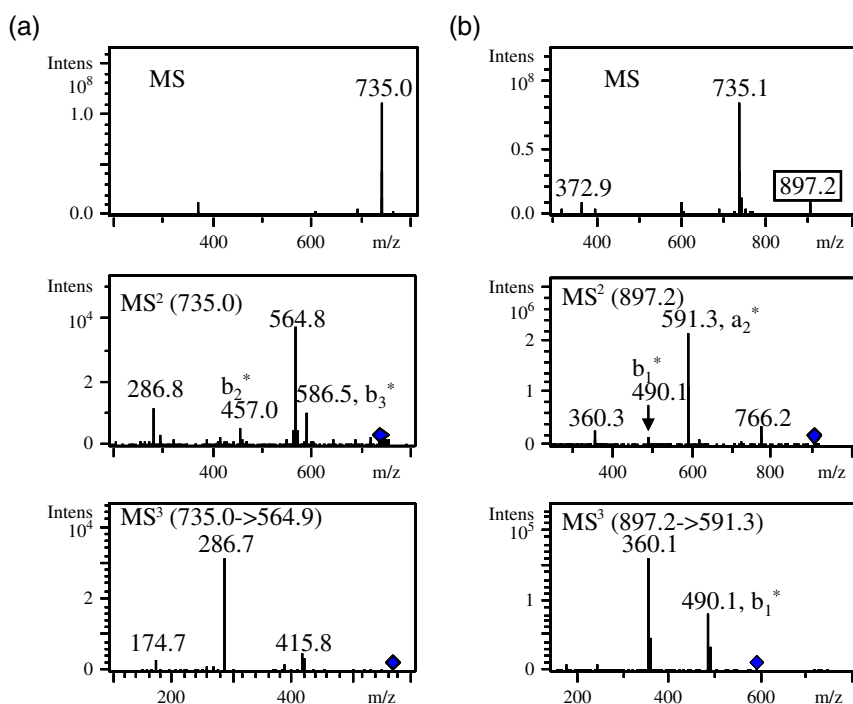


Figure 6. MSⁿ spectra of houttuynin-modified FEEM. (a) Schiff base adduct (P1, m/z 735.0); (b) pyridinium adduct (P2, m/z 897.2).

LC/MSⁿ analysis of P4 gave the molecular ion at m/z 990.3, which was 342 Da higher than that of peptide IVTNTT standard, indicating the addition of two molecules of houttuynin to peptide IVTNTT with a loss of three water molecules (Fig. 5b). This might help us assign P4 as the pyridinium adduct derived from houttuynin with *N*-terminal IVTNTT. The MS/MS spectrum gave the fragment ions a_2^* (m/z 527.3) and pyridinium (m/z 360.3). The MS³ data of the m/z 527.3 ($990.3 \rightarrow 527.3$) showed fragment ions of b_1^* (m/z 456.1) and pyridinium (m/z 360.1) (Fig. 7b).

Identification of tryptic peptides from houttuynin-HSA adducts

Tryptic digestion of the modified and blank HSA samples creates a large number of peptides. To facilitate the evaluation of the peptides, the digested mixtures were analyzed using the LC/MSⁿ system. Peptides featuring the addition of 184 and 342 Da or their multiples, corresponding to one or several reduced Schiff bases or pyridinium adducts, were subjected to MS/MS for identification and confirmation of their modification sites. As observed, the *b* and/or *y* series ions were the most prominent

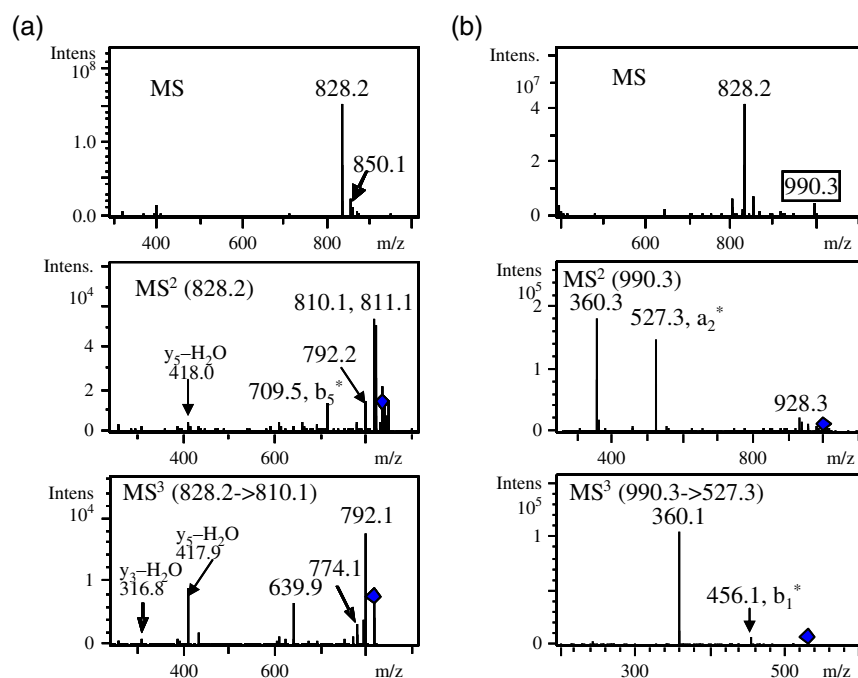


Figure 7. MSⁿ spectra of houttuynin-modified IVTNTT. (a) Schiff base adduct (P3, *m/z* 828.2); (b) pyridinium adduct (P4, *m/z* 990.3).

fragment series ions. Through these ions, the amino acid to which houttuynin was bound was determined. The typical MS/MS spectra of the adducted peptides are shown in Fig. 8. The high resolution MS data are detailed in Table 2. Annotation by an asterisk denotes the modification sites of the peptides.

Compared with data from the corresponding blank HSA sample digested, five houttuynin-adducted tryptic peptides were found. No multiple adducts were observed in this study. In each of these peptides, double- and/or triple-charged ions were found to be most abundant by ESI-MS.

Prominent [M + 2H]²⁺ (*m/z* 657.1) and [M + 3H]³⁺ (*m/z* 438.4) ions were observed in the HPLC fraction (6.8 min) and missing in the blank HSA. The ions could correspond to the miscleaved tryptic peptide KQTALVELVK (T66–67) HSA, with an additional mass of 184 Da. In the MS/MS spectrum of the ion at *m/z* 657.1, the b₁^{*} (*m/z* 313.3), b₂^{*} (*m/z* 441.3), b₄^{*}-b₉^{*} (*m/z* 613.4, 726.5, 825.6, 954.6, 1067.7, 1166.8), b₇^{*2+} (*m/z* 477.9), and b₈^{*2+} (*m/z* 534.5) ions all had *m/z* (184 + b_n^{z+})/*z*, where b_n^{z+} is the analogous b ion of the native peptide with charge *z*. Moreover, b₅^{*} - H₂O (*m/z* 708.5), b₈^{*} - H₂O (*m/z* 1049.7) and b₉^{*} - H₂O (*m/z* 1148.7) ions were also observed, providing evidence that the only way b ions can have increased *m/z* is through the modification of the lysine residue (Lys525). In addition, partial a- and y-series ions (a₅^{*}, a₈^{*}, a₉^{*}, y₃, y₄, y₇, y₈) were also observed in the MS/MS spectrum. The fragment ions confirmed the identification of the peptide as K^{*}QTALVELVK, indicating that Lys-525 is the most likely adduction site.

Identification of other houttuynin-adducted tryptic peptides was performed:

- Peptide T29–30 (AFK^{*}AWAVAR, [M + 2H]²⁺ at *m/z* 602.5 and [M + 3H]³⁺ at *m/z* 402.0). The MS/MS spectrum of *m/z* 602.5 showed b₃^{*} - H₂O, b₃^{*} - NH₃, b₅^{*}-b₈^{*} and a₆^{*} - a₈^{*}, together with their related dehydrated or deammoniated ions (b₆^{*} - H₂O, b₆^{*} - NH₃, b₇^{*} - H₂O, b₈^{*} - H₂O,

a₆^{*} - H₂O), which were modified with an additional mass of 184 Da, suggesting that Lys-212 is the adducted amino acid. The observed y₃ - NH₃, y₆, y₇^{*} and y₇^{*2+} ions could also confirm the modification sites.

- Peptide T52–53 (K^{*}VPQVSTPTLVEVSR, [M + 2H]²⁺ at *m/z* 912.9 and [M + 3H]³⁺ at *m/z* 608.9). The triple-charged ion [M + 3H - 2H₂O]³⁺ at *m/z* 597.0 was identified, and found to be very strong in the MS/MS spectrum at *m/z* 608.9. The b₂^{*}, b₄^{*}, b₅^{*}, b₇^{*}, b₁₀^{*2+}, b₁₃^{*2+} and b₁₄^{*2+} ions were observed, with an additional mass of 184 Da, indicating that Lys-414 was adducted.
- Peptide T52–53 (K^{*}VPQVSTPTLVEVSR, [M + 2H]³⁺ at *m/z* 661.3). The N-terminal sequence was identified with b₂^{*}, b₄^{*}, b₇^{*}, b₁₁^{*2+} and b₁₃^{*2+} ions with an additional mass of 342 Da. Lys-414 was suggested to be the site of adduction. The y₃, y₅, y₆, y₈, and y₉ ions confirmed the C-terminal part of the peptide.
- Peptide T54–55 (NLGK^{*}VGSK, [M + 1H]²⁺ at *m/z* 572.7 and [M + 2H]³⁺ at *m/z* 382.4). In the MS/MS spectrum of *m/z* 572.7, the double-charged ion [M + H - NH₃]²⁺ was very strong at *m/z* 564.4. The a₄^{*}, a₅^{*}, b₄^{*}, b₅^{*}, b₇^{*}, b₈^{*}, and b₈^{*} - H₂O ions were observed with an additional mass of 342 Da, making Lys-432 the adduction site. Moreover, y₃, y₄, y₆^{*}, y₆^{*2+} and y₇^{*2+} ions could also be observed in the MS/MS spectrum.

Modifications at residues Lys-212, Lys-414, and Lys-525 primarily involved the formation of Schiff base adducts; those at Lys-414 and Lys-432 were modified through the pyridinium pathway. The difference may be associated with the pK_a values of lysine residues and accessible surface areas (Wa *et al.*, 2007). Major modification sites were clustered in subdomains IIA and IIIA on the HSA sequence, which are the two principal binding regions for many small-molecule drugs (Qiu *et al.*, 1998; Sudlow *et al.*, 1976).

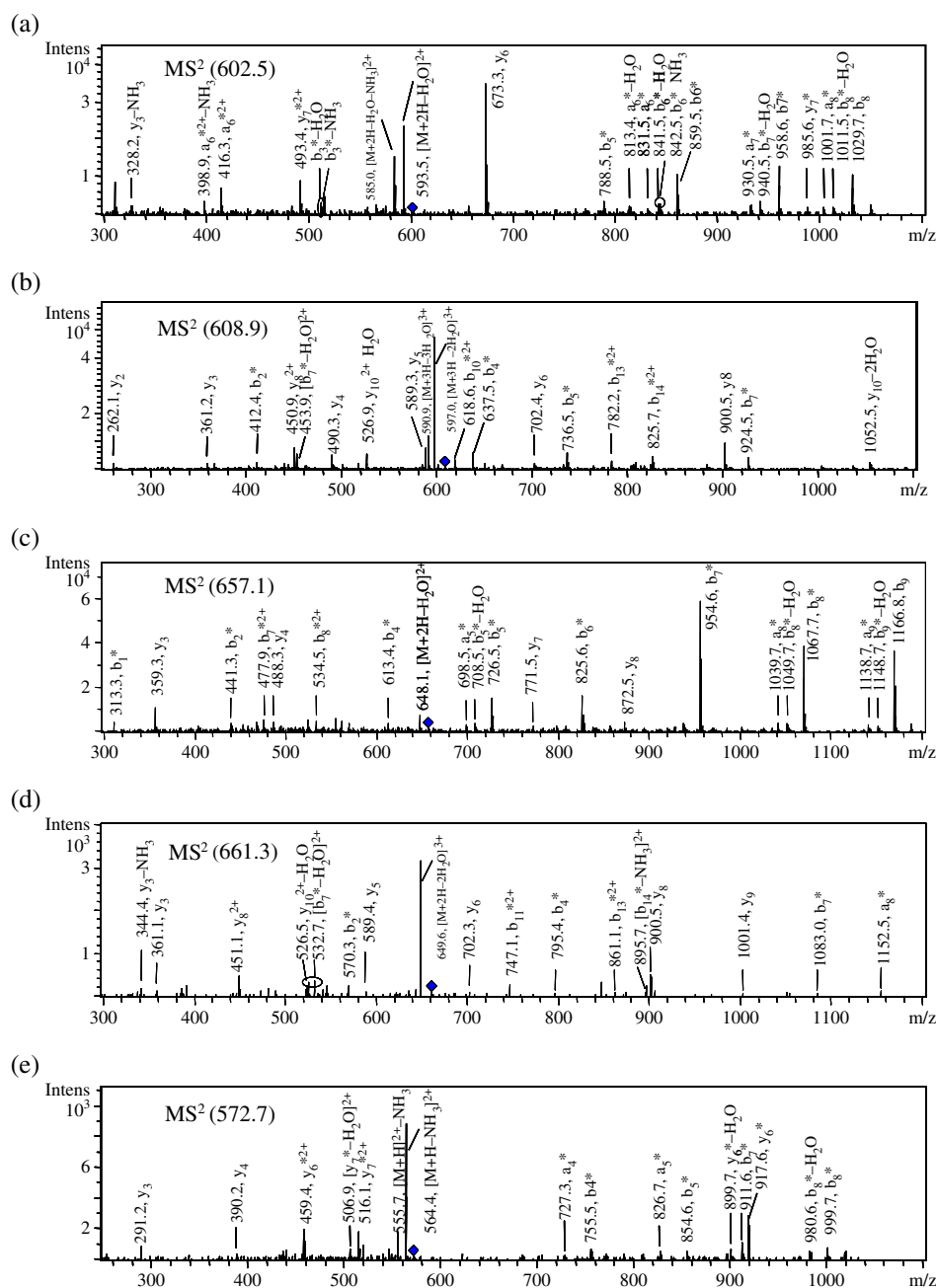


Figure 8. MS/MS spectra of the tryptic digest of houttuynin-modified HSA. (a) AFK*AWAVAR (212 – 221), $[M + 2H]^{2+}$ m/z 602.5; (b) K*VPQVSTPTLVEVSR (414 – 428), $[M + 3H]^{3+}$ m/z 608.9; (c) K*QTALVELVK (525 – 534), $[M + 2H]^{2+}$ m/z 657.1; (d) K*VPQVSTPTLVEVSR (414 – 428), $[M + 2H]^{3+}$ m/z 661.3; (e) NLGK*VGSK (429 – 436), $[M + 1H]^{2+}$ m/z 572.7. The a, b, and y fragment ions are labeled accordingly. * Modification sites.

Identification of tryptic peptides from houttuynin-Hb adducts

Compared with data from the corresponding blank Hb sample digested, three houttuynin-adducted tryptic peptides were found (Fig. 9 and Table 2). Identification of the modification site of a houttuynin adduct on the peptides will be described briefly, and only the crucial y and b series ions are listed.

- Peptide $\alpha 1-7$ (V*LSPADK, $[M + 1H]^+$ at m/z 913.5 and $[M + 2H]^{2+}$ at m/z 457.5). The N-terminal sequence was identified with b_1^* , b_2^* , b_3^* , b_5^* , b_6^* , $b_6^* - H_2O$, a_1^* , $a_1^* - H_2O$ and a_2^* ions with an additional mass of 184 Da. The observed y_2 ,

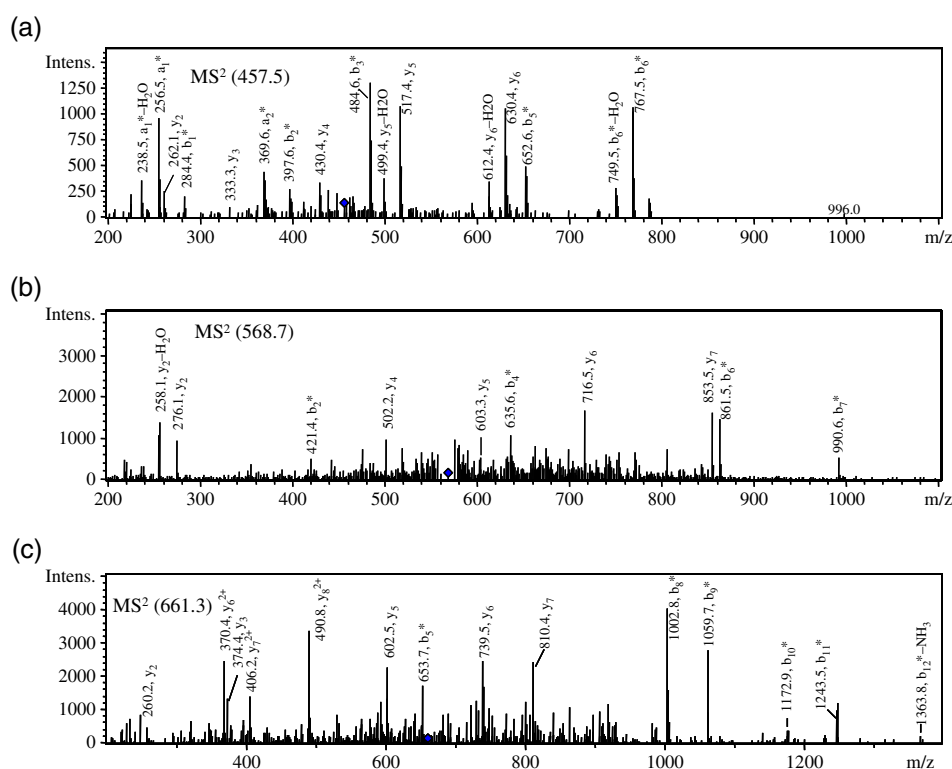
y_3 , y_4 , y_5 , $y_5 - H_2O$, y_6 and $y_6 - H_2O$ ions further confirmed the modification sites from N-terminal valine.

- Peptide $\beta 1-8$ (V*HLTPEEK, $[M + 1H]^+$ at m/z 1136.5 and $[M + 2H]^{2+}$ at m/z 568.7). The N-terminal sequence was identified with b_2^* (m/z 421.4), b_4^* (m/z 635.6), b_6^* (m/z 861.5) and b_7^* (m/z 990.6) ions with an additional mass of 184 Da. The N-terminal Val-1 was suggested to be the site of adduction. The y_2 , $y_2 - H_2O$, y_4 , y_5 , y_6 and y_7 ions confirmed the C-terminal part of the peptide.
- Peptide $\beta 66-82$ (K*VLGAFSDGLAHLNLIK, $[M + 3H]^{3+}$ at m/z 661.3). The MS/MS spectrum of m/z 661.3 showed b_5^* , b_8^* , b_9^* , b_{10}^* , b_{11}^* , and $b_{12}^* - NH_3$ ions, which were

Table 2. Summary of the peptides from trypsin digest of houttuynin-modified HSA and Hb

	Peptides	Mass to charge ratio (m/z)	Δm (Da)	Modification sites	Adduct types
HSA	T29–30	602.5 ^b , 402.0 ^c	184	Lys-212	Schiff base
	T52–53	912.9 ^b , 608.9 ^c	184	Lys-414	Schiff base
	T66–67	657.1 ^b , 438.4 ^c	184	Lys-525	Schiff base
	T52–53	661.3 ^c	342	Lys-414	pyridinium
	T54–55	572.7 ^b , 382.4 ^c	342	Lys-432	pyridinium
Hb	α 1–7	913.5 ^a , 457.5 ^b	184	Val-1	Schiff base
	β 1–8	1136.5 ^a , 568.7 ^b	184	Val-1	Schiff base
	β 66–82	661.3 ^c	184	Lys-62	Schiff base

Δm^3 (Da) is the increase in weight against the native peptides.
 a , b and c represent the single, double and triple charged ions.
Hb, hemoglobin; HSA, human serum albumin.

**Figure 9.** MS/MS spectra of the tryptic digest of houttuynin-modified Hb. (a) V*LSPADK (α 1–7), $[M + 2H]^{2+}$ m/z 457.5; (b) V*HLTPPEK (β 1–8), $[M + 2H]^{2+}$ m/z 568.7; (c) K*VLGAFSDGLAHLNLK (β 66–82), $[M + 3H]^{3+}$ m/z 661.3. The a , b , and y fragment ions are labeled accordingly. * Modification sites.

modified with an additional mass of 184 Da, suggesting that Lys-62 is the adducted amino acid. The observed y_2 , y_3 , y_5 , y_6 , y_6^{2+} , y_7 and y_7^{2+} ions could also confirm the modification sites.

In tryptic digested peptide mixtures from houttuynin-Hb adducts, only Schiff base adducts with N -terminal and lysine amines were detected, and pyridinium adducts with amino amines were not found. There are two probable reasons for this. First, the amount of pyridinium adducts formed might be much less than that of Schiff base adducts, directly resulting in detection difficulties in measurable concentration via the LC/MSⁿ analysis. Second, Hb is structurally different from HSA, which might react with houttuynin to form only Schiff base adducts.

Conclusions

In summary, houttuynin, a representative β -keto aldehyde, was demonstrated to adduct with lysine amines and the N -terminal α -NH₂ group of HSA and Hb under physiological conditions. The modification site(s) and structure(s) of adducts were determined by enzymatic digestion experiment and by LC/MSⁿ analysis. The results revealed that houttuynin-modified HSA adducts were formed mainly at Lys-212, Lys-414 and Lys-525 for Schiff base adducts, and at Lys-414 and Lys-432 for pyridinium adducts, and houttuynin-modified Hb adducts at Val-1 of the α - and β -chains and at Lys-62 of the β -chain for Schiff base adducts. The covalent binding of proteins is speculated to be responsible for the adverse reactions induced by herba houttuyniae injection.

SUPPORTING INFORMATION

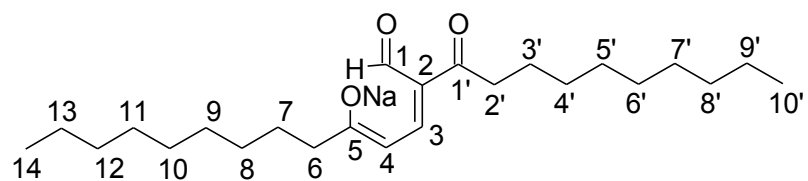
Supporting information can be found in the online version of this article.

Acknowledgments

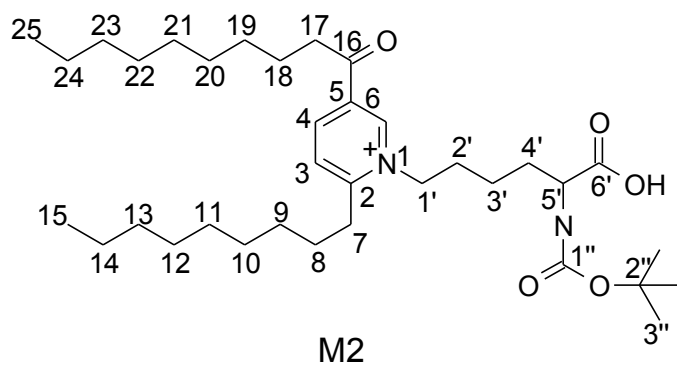
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References

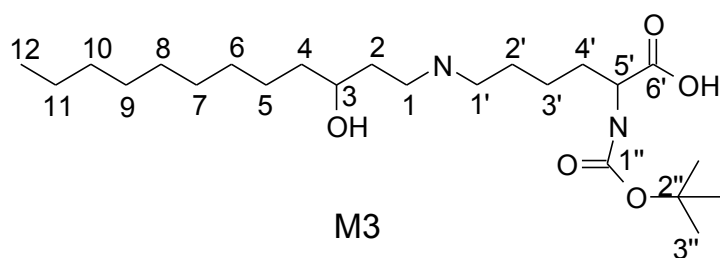
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Houttuynin dimer



M2



M3

Figure S1. The structures of M2, M3 and houttuynin dimer.

Table S1. The ^1H NMR and ^{13}C NMR data of Houttuynin dimer.

No.	Houttuynin dimer	
	δ_{H}	δ_{C}
1	9.54 (1H, s)	186
2	—	113
3	7.94 (1H, d, J = 15.6 Hz)	142
4	7.20 (1H, d, J = 15.6 Hz)	117
5	—	205
14 or 10'	0.904 (6H, t, J = 6.6Hz)	13.0
1'	—	200
2'	2.63 (2H, t, J = 8.0 Hz)	38.9

No.	M2		M3	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1			3.12 (2H, m)	49.1
2	–	164		
3	8.13 (1H, d, J = 8.1 Hz)	131	3.68 (1H, m)	71.1
4	8.89 (1H, dd, J = 8.1 Hz, 1.5 Hz)	145		
5	–	136		
6	9.45 (1H, d, J = 1.5 Hz,)	148		
12			0.894 (3H, t, J = 6.6Hz)	14.9
15	0.900 (3H, m)	14.9		
16	–	199		
25	0.900 (3H, m)	14.9		
1'	4.96 (2H, t, J = 8.1 Hz)	59.9	2.99 (2H, t, J = 7.7Hz)	47.3
5'	4.06 (1H, m)	56.3	4.00 (1H, m)	56.5
6'	–	179	–	179
1''	–	158	–	158
2''	–	80.5	–	80.7
3''	1.68 (9H, s)	29.3	1.44 (9H, s)	29.3

Table S2. The ^1H NMR and ^{13}C NMR data of M2 and M3.

