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Isolation and Identification of Phase 1 Metabolites of Demethoxycurcumin in Rats

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d) Abbreviations: HPLC, high-performance liquid chromatography; PTLC, performance thin layer chromatography; TLC, thin layer chromatography; EtOH, ethanol; MeOH, methanol; 2D NMR, two-dimensional nuclear magnetic resonance; HMQC, heteronuclear multiple-quantum correlation; HMBC, heteronuclear multiple-bond correlation; ESI-MS, electrospray ionization-mass spectrometry; LC/MS^n, liquid chromatography-mass spectrometry at stage n.

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Abstracts: Curcuminoids are a safe natural food coloring additive with anti-inflammatory, antioxidant and anticarcinogenic activities. Although demethoxycurcumin is one of the major bioactive constituents of curcuminoids, knowledge about its metabolic fate is scant. In the present study, four new metabolites: 5-dehydroxy-hexahydro-demethoxycurcumin-A (**M-1**), 5-dehydroxy-hexahydro-demethoxycurcumin-B (**M-2**), 5-dehydroxy-octahydro-demethoxycurcumin-A (**M-3**) and 5-dehydroxy-octahydro-demethoxycurcumin-B (**M-4**) were isolated from feces of male Wistar-derived rats and from urine, three new metabolites: 5-O-methyl-hexahydro-demethoxycurcumin-A (**M-7**), 5-O-methyl-hexahydro-demethoxycurcumin-B (**M-8**), and 5-dehydroxy-dihydro-demethoxycurcumin-B (**M-9**) and two known metabolites: hexahydro-demethoxycurcumin-A (**M-5**), hexahydro-demethoxycurcumin-B (**M-6**) were isolated. Their structures were established by chemical and spectral methods. All of them were reductive metabolites. Possibly of greater importance is that they occurred as pairs of isomers with a methoxyl group substituted on a different benzene ring. This finding in the metabolism of curcuminoids is reported here for the first. In addition, the 5-dehydroxy or 5-O-methylated metabolites are also novel finding. The fact that the metabolites occurred as pairs of the isomers suggests that demethoxycurcumin possibly undergoes tautomerization between 3-keto-5-enol (form A) and 3-keto-5-enol (form B) (Fig. 1) in rats. On the basis of the metabolite profiles, metabolic pathways of demethxoycurcumin in rats are proposed.

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Introduction

Curcuminoids are natural yellow pigments and food-coloring agents present in the rhizomes of the Asian tropical plant *Curcuma longa* which has been used as a traditional medicinal herb for thousands of years. The dried rhizome of *C. longa* has been widely used as an aromatic stomachic, carminative, anthelmintic, laxative, and as condiments in foods as well as for liver ailment (Nurfina et al., 1997). Curcuminoids are responsible for its biological actions. Curcuminoids, consist mainly of three diarylheptanoids: curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Govindaajan et al., 1980) (Fig. 2). These are recognized for their beneficial effects such as a choleretic (Ramprasad et al., 1956; Hermann et al., 1991), as anti-oxidants (Unnikrishnan et al., 1995; Sharma, 1976), anti-inflammatory agents (Arora et al., 1971; Ghatak et al. 1972), for treating human immunodeficiency virus infections (Mazumber et al., 1995; Eigner et al., 1999) and as anticarcinogens (Araujo et al., 2001; Duvoix et al. 2005; Kuttan et al., 1985; Conney et al. 1991). In recent years, their ability to protect neuronal cells from βA insult (Park et al., 2002; Kim et al., 2001) has also attracted great attention. Demethoxycurcumin was found to be the more effective in protecting PC12 and HUVEC cells from βA insult than curcumin. Although numerous aspects of the pharmacology of curcuminoids, in particular its activity as chemopreventive agent, have been studied, their metabolism in humans and experimental animals has not been fully characterized. The metabolism of curcumin has been studied mostly in rats in vivo and in vitro (Holder et al., 1978; Ravindranath et al., 1982; Asai et al.,1999; Pan et al., 2000; Ireson et al., 2001; Ireson et al., 2002). More recently, information on the metabolism of curcumin in humans has been obtained from in vitro studies with hepatic and intestinal cells and subcellular fractions (Ireson et al., 2001; Ireson et al., 2002), as well as from clinical studies in cancer patients (Cheng et al., 2001; Sharma et al., 2001; Garcea et al., 2004; Sharma et al., 2004).

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The metabolism of demethoxycurcumin, which is the major active component in curcuminoids such as curcumin, has only been studied on one report. In that investigation, in vitro studies with tissue slices and subcellular fractions from rat liver were reported (Hoehle et al., 2006). No data have yet been published on the metabolism of demethoxycurcumin in vivo. Therefore, studies of the metabolic products of demethoxycurcumin in feces and urine after oral administration in male Wistar rats were undertaken. The isolation and identification of nine phase 1 reductive metabolites of demethoxycurcumin are described here.

Materials and Methods

Materials. Dry rhizomes of *C. Longa* were collected from Gui Zhou province, China. A voucher specimen was identified by Prof. *Qi-Shi Sun*(No.CL200209) and deposited at the Department of Natural Products Chemistry, Shenyang Pharmaceutical University, China.

Demethoxycurcumin. Dry rhizomes of *C. Longa*(2.5kg) were pulverized then extracted 3 times for 0.5hr/each time by ultrasound in an eight-fold volume (wt/vol) of 80% EtOH. The EtOH solutions were combined and condensed to yield 362g. Then the extract was chromatographed on a silica gel column using a $CHCl_3$-MeOH gradient solvent system to yield 17 fractions (Fr.A-Q). Fr.E (25.5g) was further subjected to column chromatography on a silica gel with $CHCl_3$: MeOH 50:1 to yield 4 fractions (Fr.F1-Fr.F4). Demethoxycurcumin (6.8g) was obtained as a yellow-orange amorphous powder from Fr.F4 after repeated precipitation in MeOH. It had a purity of >98% according to HPLC analysis. The structure of demethoxycurcumin was identified and confirmed by comparing the MS and ^{1}H and ^{13}C NMR spectral data with those previously reported (Kiuchi et al., 1993).

Demethoxycurcumin: a yellow-orange amorphous powder, ESI-MS: m/z 337 $[M-H]^-$. ^{1}H-NMR

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(300MHz, DMSO-*d6*) and ^{13}C-NMR (75MHz, DMSO-*d6*) (Table 5).

Chemicals The purity of MeOH for HPLC was 99.9% was from Jiangsu Hanbang Chemical Factory (Jiangsu, China), silica gel for column chromatography (200–300 mesh), and silica gel G_{60} for thin-layer chromatography (300-400mesh), preparative thin-layer chromatography (300-400mesh) and macroporous resin D101 from Qingdao Marine Chemical Factory (Shandong, China), reverse-phase preparatory TLC from Merck Co. Sephadex LH-20 and ODS from Pharmacia Company.gy Co. Ltd. Other chemicals were analytical grade, provided by Shenyang Chemical reagent factory (Shenyang, China).

Animals Male Wistar-derived rats (200-250g) were provided by the Institute of Jingfeng Medical Animal Center (Beijing, China). Subjects were judged to be in good health and housed in conditions of temperature-(22±2℃), humidity-(55±10%), and light-(8:00-20:00) in a controlled breeding room where they were acclimated for 7 days prior to study. Normal food and water were available ad libitum, but withdrawn 24hr prior to intragastric administration of demethoxycurcumin. Demethoxycurcumin was orally administered as 30% aq. 1,2- propylene glycol solution. Urine and feces were collected for 48 hr. from animals housed in stainless steel metabolism cages equipped with a urine and feces separator.

Preliminary Studies For the sample group (four rats), a solution of demethoxycurcumin (50mg/kg) was administered orally by direct stomach intubation in a volume of 10ml/kg body weight. For the control group (four rats), the solvent 30% aq. 1,2-propylene glycol only was orally administered in rats by the same method. Pooled urine and feces of the sample group and those of the control group were simultaneously treated with parallel procedures. Urine was subjected to macroporous resin D101 chromatography and eluted with H_2O, 50% EtOH and 95% EtOH in turn after filtration. Each elution

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was concentrated to nearly 1.0ml in vacuo and detected by T.L.C in $CHCl_3$: MeOH (15:1) and $CHCl_3$: MeOH: H_2O (7:3:0.5), and spraying with 10% H_2SO_4. After heating, two metabolite spots [R_F 0.45 (spot 1) and 0.50 (spot 2)] were observed in 50% EtOH and 95% EtOH fraction of the sample group in $CHCl_3$: MeOH (15:1) and two metabolite spots were observed at R_F 0.05 and 0.40 in 50% EtOH fraction of the sample group in $CHCl_3$: MeOH: H_2O (7:3:0.5), but not in those of the control group. Feces were extracted with EtOAC (100ml) then MeOH (100ml) for 2hr. for twice. The combined EtOAC and MeOH extracts were concentrated to nearly 1.0ml under vacuum, then detected by T.L.C in $CHCl_3$: MeOH (15:1) and $CHCl_3$: MeOH: H_2O (7:3:0.5), and spraying with 10% H_2SO_4. After heating, the same metabolite spot [R_F 0.57(spot 3)] was observed both in EtOAC and MeOH extracts of the sample group and one metabolite spot [R_F 0.40(spot 4)] was observed in MeOH extracts of the sample group in $CHCl_3$: MeOH (15:1), but not in those of the control group. There was not other metabolite spot observed in $CHCl_3$: MeOH: H_2O (7:3:0.5).

Isolation of Metabolites A solution of demethoxycurcumin (6mg/ml) was orally administered at 50mg/kg body weight to eighty rats and then repeated at one week interval. The total administration of demethoxycurcumin was 3g. The urine (approximately 10,000 ml in total) and feces (approximately 385g) were treated by the same methods used in the preliminary tests. The results in T.L.C were essentially identical with those in the preliminary tests. The EtOAC (31.5g) and MeOH (21.0g) extracts of the feces were chromatographed, respectively, on silica gel columns using a $CHCl_3$-MeOH gradient solvent system to yeild 8 (FE1-8) and 14 fractions (FM1-14). FE1 and FM1 (containing spot 3) were combined and subjected to Sephadex LH-20 column chromatography ($CHCl_3$: MeOH 1:1) followed by C_{18}-ODS reverse-phase open column chromatography (60% MeOH in water) then purified by PTLC ($CHCl_3$: MeOH 15:1) to yield spot 3 (the mixture of **M-1** and **M-2**, 40.3mg). FM 3 (containing spot 4)

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was applied to Sephadex LH-20 column chromatography ($CHCl_3$: MeOH 1:1) followed by C_{18}-ODS reverse-phase open column chromatography (60% MeOH in water) then purified using Sephadex LH-20 column chromatography eluting with MeOH to afford spot 4 (the mixture of **M-3** and **M-4**, 3.1mg). The 50% EtOH fraction (26g) of the urine was dissolved in MeOH then filtered. The filtrate (containing spot 1 and 2) was subjected to Sephadex LH-20 column chromatography, eluted with MeOH followed by C_{18}-ODS reverse-phase open column chromatography (60% MeOH in water) to yield 7 fractions (U01-07). Further purification of U05 (containing spot 1 and 2) was performed using C_{18}-ODS reverse-phase preparative HPLC (55% MeOH in water, 277nm) to afford 5 fractions (U051-U055). U055 was further subjected to C_8-ODS reverse-phase preparative HPLC (50% MeOH in water, 284nm) to yield **M-9** (1.8mg). U051 (containing spot 1 and 2) was applied to PTLC ($CHCl_3$: MeOH 15:1) to yield spot 1 (the mixture of **M-7** and **M-8**, 17.3mg) and spot 2 (the mixture of **M-5** and **M-6**, 67.6mg).

Spectroscopic Methods NMR spectra were measured on Bruker ARX-300 or AV-600 spectrometers, using TMS as an internal standard. Electrospray ion trap mass spectrometry was performed on a Agilent 1100 Series LC/MSD Trap instrument whose mass range is 50 to 5000 (the mass was calibrated). The instrument was operated in the both positive and negative ion modes, using nitrogen for nebulizing and dry gas. The ionization was performed applying the following parameters: dry gas temperature, 300℃; dry gas rate: 5L/min; spray voltage, 4000V, atomization, 15psi. Sample solutions were directly introduced into the ESI source at a flow rate of 3uL/min by a syringe pump.

HPLC Instruments Preparative HPLC was performed using a C-8 column (C-8, 250×20 mm, Inertsil Pak) and a C-18 column(C-18, 250×20 mm, Inertsil Pak) in a Waters 600 liquid chromatograph apparatus equipped with a Waters 490 UV detector (Waters, Milford, MA). Analytical HPLC was

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performed using a C-18 column (C-18, 25×20 mm, Inertsil Pak) in a Waters 600 liquid chromatograph apparatus equipped with a Waters 996 UV detector.

Results

Metabolites M-1 and M-2 were obtained together as a viscous oil. The positive ESI-MS showed two quasi-molecular ion peaks at *m/z* 329 ($[M+H]^+$) and 351 ($[M+Na]^+$), and the negative ESI-MS gave a quasi-molecular ion peaks at *m/z* 327 ($[M-H]^-$). The ^{13}C and ^1H-NMR spectra revealed two extremely similar groups of signals, which suggested that there were a pair of isomers with the same molecular formula of $C_{20}H_{24}O_4$. For the two groups of signals, the stronger one corresponded to **M-2** and the weaker one corresponded to **M-1**. The ^{13}C and ^1H-NMR and HMQC spectra displayed two sets of 1,3,4-trisubstituted benzene ring signals [δ6.62 (1H, brd, *J*=8.0Hz), 6.64 (1H, d, *J*=1.5Hz), 6.81(1H, d, *J*=8.0Hz) and δ6.62 (1H, brd, *J*=8.0Hz), 6.66 (1H, d, *J*=1.5Hz), 6.81 (1H, d, *J*=8.0Hz)], two sets of 1,4-bis-substituted benzene ring signals [δ6.74 (2H, d, *J*=8.4Hz), 6.99 (2H, d, *J*=8.4Hz) and δ6.74 (2H, d, *J*=8.4Hz), 6.97(2H, d, *J*=8.4Hz)], two methoxy groups [δ3.84 (3H, s, OCH_3) and δ3.82 (3H, s, OCH_3)], two overlapped carbonyl carbonyl signals (δ211.2) and twelve methylene groups, which suggested that **M-1** and **M-2** were the reduced metabolites of olefinic C-C double bonds of demethxoycurcumin. In the HMBC spectrum of **M-2** (Fig. 3), correlations from H-2′, H-6′ (δ6.99) to C-4′ (δ154.1), C-3′and C-5′ (δ115.3); H-3′, H-5′ (δ6.74) to C-1′ (δ132.7) and C-4′ (δ154.1) indicated the presence of 4′-hydroxyphenyl (group A). Correlations from H-2″ (δ6.64) to C-3″ (δ146.3), C-4″ (δ143.5) , C-5″ (δ114.1) and C-6″ (δ120.8); H-5″ (δ6.81) to C-1″ (δ134.2), C-2″ (δ111.0), C-3″ (δ146.3), C-4″ (δ143.5), H-6″ (δ6.62) to C-2″ (δ111.0), OCH_3 (δ3.84) to C-3″ (δ146.3) suggested the existence of 3″-methoxy-4″-hydroxyphenyl (group B). Correlations from H-1 (δ2.80) to C-2 (δ44.5)

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and C-3 (δ211.2); H-2 (δ2.67) to C-3 (δ211.2); H-4 (δ2.39) to C-3 (δ211.2) and C-5 (δ23.3); H-5 (δ1.57) to C-3 (δ211.2), C-4 (δ42.9) and C-7 (δ35.3); H-6 (δ1.51) to C-4 (δ42.9), C-5 (δ23.3) and C-7 (δ35.3); H-7 (δ2.50) to C-5 (δ23.3) and C-6 (δ31.1) confirmed the presence of the moiety of 3-heptanone (group C). In addition, correlations from H-2′, H-6′ (δ6.99) to C-1 (δ28.9) justified the connectivity between the group A and group C at C1′/C1, and correlations from H-2″ (δ6.64) and H-6″ (δ6.62) to C-7 (δ35.3) revealed the junction between the group B and group C at C1″/C7 (Fig. 7). Thus, **M-2** was elucidated as 1-(4′-hydroxyphenyl)-7-(3″-methoxy-4″-hydroxyphenyl)-3-heptanone (namely 5-dehydroxy-hexahydro-demethoxycurcumin-B). By the same methods, the assignment of 3′-methoxy-4′-hydroxyphenyl moiety (group A) in **M-1** was confirmed by HMBC correlations of H-2′ (δ6.66) with C-3′ (δ146.4), C-4′ (δ143.8) , C-5′ (δ114.3) and C-6′ (δ120.7); H-5′ (δ6.81) with C-1′ (δ132.9), C-2′ (δ111.1), C-3′ (δ146.4), C-4′ (δ143.8), H-6′ (δ6.62) with C-2′ (δ111.1) and protons of methoxy (δ3.82) with C-3′ (δ146.4). The assignment of 4′-hydroxyphenyl moiety (group B) in **M-1** was supported by the HMBC correlations of H-2″, H-6″ (δ6.97) with C-4″ (δ153.8), C-3″and C-5″ (δ115.1) and H-3″, H-5″ (δ6.74) with C-1″ (δ134.0) and C-4″ (δ153.8). The presence of 3-heptanone moiety (group C) in **M-1** was suggested by the HMBC correlations of H-1 (δ2.80) with C-2 (δ44.6) and C-3 (δ211.2); H-2 (δ2.67) with C-3 (δ211.2); H-4 (δ2.39) with C-3 (δ211.2) and C-5 (δ23.2); H-5 (δ1.57)with C-3 (δ211.2), C-4 (δ42.9) and C-7 (δ34.7); H-6 (δ1.51) with C-4 (δ42.9), C-5 (δ23.2) and C-7 (δ34.7) and H-7 (δ2.50) with C-5 (δ23.2) and C-6 (δ31.1). Futhermore, the connectivity between the group A and group C at C1′/C1 was justified by the HMBC correlations of H-2′ (δ6.66) with C-1 (δ29.5), and the junction between the group B and group C at C1″/C7 demonstrated by the correlations of H-2″, H-6″ (δ6.97) with C-7 (δ34.7). Thus, **M-1** was identified as 1-(3′-methoxy-4′-hydroxyphenyl)-7-(4″-hydroxyphenyl)-3-heptanone (namely 5-dehydroxy-

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hexahydro-demethoxycurcumin-A).

Metabolites M-3 and M-4 were obtained together as a viscous oil. The positive ESI-MS showed a quasi-molecular ion peak at *m/z* 353 ($[M+Na]^+$) and the negative ESI-MS gave a quasi-molecular ion peaks at *m/z* 329.0 ($[M-H]^-$). The ^{13}C and ^1H-NMR spectra also revealed two extremely similar groups of signals which suggested that there were a pair of isomers with the same molecular formula of $C_{20}H_{26}O_4$. For the two groups of signals, the stronger one corresponded to **M-4** and the weaker one corresponded to **M-3**. The ^{13}C and ^1H- NMR data of **M-4** were similar to those of **M-2** except for the upfield shifts of C-3 by 139.9ppm and the appearance of the H-3 [δ3.62(1H, m)], which showed that **M-4** was the 3-hydroxy reductive product of **M-2**. In addition, the ^{13}C and ^1H-NMR data of **M-4** were nearly identical with those previously reported (Li et al., 2004). Thus, **M-4** was established as 1-(4′-hydroxyphenyl)-7-(3″-methoxy-4″-hydroxyphenyl)-3-heptitol (namely 5-dehydroxy-octahydro-demethoxycurcumin-B). The chemical shifts of **M-3** were similar to those of **M-1** except for those around C-3. In the ^{13}C-NMR spectrum, the signal of C-3 was shifted to higher field (δ71.4) compared with that of **M-1** (δ211.2). In the ^1H-NMR spectrum, the signal of H-3 [δ3.62(1H, m)] occurred. These results suggested that **M-3** was the 3-hydroxy reductive product of **M-1**. Therefore, **M-3** was established as 1-(3′-methoxy-4′-hydroxyphenyl)-7-(4″-hydroxyphenyl)-3-heptitol (namely 5-dehydroxy-octahydro-demethoxycurcumin-A).

Metabolites M-5 and **M-6** were obtained together as a viscous oil. The positive ESI-MS showed a quasi-molecular ion peak at *m/z* 367 ($[M+Na]^+$) and the negative ESI-MS gave a quasi-molecular ion peak at *m/z* 343 ($[M-H]^-$). Similarly, the ^{13}C and ^1H-NMR spectra also revealed two extremely similar groups of signals which suggested that there were a pair of isomers with the same molecular formula of $C_{20}H_{24}O_5$. For the two groups of signals, the stronger one corresponded to **M-6** and the weaker one

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corresponded to **M-5**. In the ^{1}H and ^{13}C-NMR spectra, the signal pattern of **M-5** and **M-6** was nearly identical with that of **M-1** and **M-2**, except that the signals of C-5 and H-5 of **M-5** were shifted to lower fields [δ_C66.9 and δ_H4.03(1H,m)] compared with those of **M-1** [δ_C23.2 and δ_H1.57(2H,m)] and the signals of C-5 and H-5 of **M-6** were shifted to lower fields [δ_C67.0 and δ_H4.03(1H,m)] compared with those of **M-2** [δ_C23.3 and δ_H1.57(2H,m)], respectively. These results suggested that **M-1** and **M-2** were the 5-dehydroxylated products of **M-5** and **M-6**. In addition, the MS, ^{1}H and ^{13}C-NMR data of **M-5** were nearly identical with those previously reported (Kikuzaki et al., 1991) and those of **M-6** were same as those previously reported (Shin et al., 2002). Thus, **M-5** and **M-6** were determined to be hexahydro-demethoxycurcumin-A and hexahydro-demethoxycurcumin-B, respectively.

Metabolites M-7 and **M-8** were obtained together as a viscous oil. The positive ESI-MS showed a quasi-molecular ion peak at *m/z* 381 ($[M+Na]^+$) and the negative ESI-MS gave a quasi-molecular ion peaks at *m/z* 357 ($[M-H]^-$). Similarly, the ^{13}C and ^{1}H-NMR spectra also revealed two extremely similar groups of signals which suggested that there were a pair of isomers with the same molecular formula of $C_{21}H_{26}O_5$. For the two groups of signals, the stronger one corresponded to **M-8** and the weaker one corresponded to **M-7**. The chemical shifts of **M-7** were nearly the same as those of **M-5** except for the following findings: the proton signal of H-5 (δ3.71) was shifted upfield by 0.32ppm, the carbon signal of C-5 (δ76.7) was shifted downfield by 9.7ppm, and the signals of methoxy [δ_C56.9 and δ_H3.31(3H, s)] were present which indicated that **M-7** was the 5-O-methyl ether of **M-5**. Therefore, **M-7** was elucidated as 5-O-methyl-hexahydro-demethoxycurcumin-A. By the same method, **M-8** was determined to be the 5-O-methyl ether of **M-6**, and the MS, ^{1}H and ^{13}C-NMR data of **M-8** were nearly the same as those previously reported (Li et al., 2003). Therefore, **M-8** was established as 5-O-methyl-hexahydro-demethoxycurcumin-B.

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Metabolite M-9 was obtained as a yellow amorphous powder. The positive ESI-MS showed two quasi-molecular ion peaks at *m/z* 325 ($[M+H]^+$) and 347 ($[M+Na]^+$) and the negative ESI-MS gave a quasi-molecular ion peaks at *m/z* 323 ($[M-H]^-$), corresponding to the molecular formula $C_{20}H_{20}O_4$, which was further supported by the ^{1}H-NMR and ^{13}C-NMR spectral data. The ^{13}C and ^{1}H-NMR and HMQC spectra displayed a set of 1,3,4-trisubstituted benzene ring signals [δ6.76 (1H, d, *J*=8.1Hz), 6.96 (1H, brd, *J*=8.1Hz), 7.16(1H, brs)], a set of 1,4-bis-substituted benzene ring signals [δ6.64 (2H, d, *J*=8.1Hz), 7.00 (2H, d, *J*=8.1Hz)], one pair of *trans* conjugated olefinic protons [δ6.22 (1H, d, *J*=15.5Hz), 7.35 (1H, dd, *J*=10.4, 15.4Hz) and δ6.93 (1H, dd, *J*=10.2, 15.4Hz), 6.96 (1H, d, *J*=16.0Hz)], a methoxy group [δ3.80 (3H, s, OCH_3)], a carbonyl signal (δ199.2) and two methylene groups. The HMBC correlations of H-1 (δ2.71), H-2 (δ2.84), H-4 (δ6.22) and H-5 (δ7.35) with C-3 (δ199.2) suggested that H-1 and H-5 are three bonds away from the C-3 carbonyl group while H-2 and H-4 are adjacent to the carbonyl group. The HMBC spectrum revealed H-2′, 6′ (δ7.00) to be correlated with C-1 (δ29.0), and H-2″ (δ7.16) correlated with C-7 (δ141.9). Thus, **M-9** was identified as 5-dehydroxy-dihydro-demethoxycurcumin-B. It is a 5-dehydroxy product with reduction of the double bond between C-1 and C-2 of demethoxycurcumin. The chemical shifts of **M-9** were nearly identical with those previously reported (Li et al., 2004).

Discussion

This is the first study on the metabolism of demethoxycurcumin in vivo. Nine phase 1 metabolites were obtained and identified by ESI-MS spectra and NMR spectroscopy including ^{1}H-NMR, ^{13}C-NMR, and two-dimensional NMR (HMQC, HMBC). Compared with earlier reports on the metabolism of demethoxycurcumin in vitro (Hoehle et al., 2006), an identical result was obtained in that reduction of

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the aliphatic moiety is the only pathway in phase 1 metabolism and no oxidative metabolites were discovered. However, there were two new discoveries in the present study: First the existence of the dehydroxy or methylated metabolites was demonstrated and secondly the existence of the isomers with a methoxy group substituted on a different benzene ring. In this study, the major metabolites of demethoxycurcumin in urine were hexahydro-demethoxycurcumins (**M-5** and **M-6**) and the 5-O-methyl-hexahydro-demethoxycurcumins (**M-7** and **M-8**) together with traces of 5-dehydroxy-dihydro-demethoxycurcumins (**M-9**). While the major metabolites in feces were 5-dehydroxy-hexahydro-demethoxycurcumin (**M-1** and **M-2**) and 5-dehydroxy-octahydro –demethoxycurcumin (**M-3** and **M-4**). Combined with previous reports on the metabolism of curcuminoids (Hoehle et al., 2006; Holder et al., 1978; Wahlstrom et al., 1978; Ireson et al., 2001), it might be presumed that some demethoxycurcumin should initially undergo reduction to form dihydro, tetrahydro, hexahydro (**M-5** and **M-6)** and octahydro-demethoxycurcumin in a stepwise fashion (Ireson et al., 2002) followed by dehydroxylation (Bokkenheuser et al., 1981; Feighner et al., 1980; Kasahara et al., 1995) to form 5-dehydroxy-dihydro (**M-9)**, 5-dehydroxy-hexahydro (**M-1** and **M-2)** and 5-dehydroxy-octahydro-demethoxycurcumins (**M-3** and **M-4)**; on the other hand, some demethoxycurcumin might be initially methylated (Yang et al., 2005) followed by reduction to form 5-O-methyl-hexahydro-demethoxycurcumins (**M-7** and **M-8**). On the basis of the metabolite profiles, the metabolic pathways of demethoxycurcumin in rats are proposed (Fig. 4).

Based on present knowledge concerning the metabolism of curcuminoids, curcuminoids as well as their reduced metabolites appear to be easily conjugated in vivo and in vitro. The reported conjugates include monoglucuronides, monosulfates, and mixed sulfate/glucuronides (Hoehle et al., 2006; Holder et al., 1978; Ravindranath et al., 1982; Asai et al., 2000). In this study, seven new phase 1 metabolites

were discovered, which provide new types of precursors for research on phase 2 metabolites of curcuminoids. In addition, because of the low bioavailability of curcumin, some previous research suggested that the pharmacological activities of curcumin was in part mediated by its metabolites (Ireson et al., 2001; Ireson et al., 2002). This has been confirmed by recent experiments with the activities of the phase I metabolites (Murugan et al., 2006; Leyon et al., 2004; Pari et al., 2004; Limtrakul et al., 2006; Lee et al., 2005). Further studies of the nine phase 1 reductive metabolites should clarify whether they remain the activity or not.

From the comparison of the ^{13}C-NMR spectra of the four pairs of isomers, it was always found that there were two very similar groups of signals and one stronger than the other. These findings suggest that, in rats, demethoxycurcumin possibly undergoes tautomerization between 3-keto-5-enol (form A) and 3-keto-5-enol (form B) (Fig. 1) and that the forms of these two isomers were not equal, one form (form B) was the major one and that the other form (form A) was the minor one. In the HMQC spectrum of **M-9**, the proton signals δ_H 7.40 (d, 8.6Hz) associated with δ_C 129.2 (Fig. 8) and δ_H 3.76 with δ_C 55.7 were observed in addition to the proton and carbon signals of **M-9**, which could be assigned to the isomer of **M-9**. The reason that the other correlative signals of the isomer were not entirely displayed in the ^{13}C and ^{1}H-NMR spectra of **M-9** might be due to the scant amount of the compound available.

M-5 and **M-6** presumably would begin to transfer partly to **M-7** and **M-8** when placed in MeOH for more than two weeks. However, the spot 1 (the mixture of **M-7** and **M-8**), which was observed in TLC in the preliminary study and at that time, the urine sample had not yet been dealed with MeOH. The data demonstrated that **M-7** and **M-8** were the actual metabolites of demethoxycurcumin in rats. However, a portion of **M-7** and **M-8** probably transfered from **M-5** and **M-6** because a large quantity of

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MeOH was utilized during the course of the subsequent separation.

Structural elucidation of metabolites is an important task in drug metabolism studies. In recent years, comparisons of ESI-MS^n data and retention times in HPLC with synthesized standards are usually used to identify the structures of metabolites. However, the structures of some metabolites deduced only from LC/MS^n data might not be correct, especially in the case of the existence of isomerism of the metabolites. In this study, four groups of isomers (**M-1** and **M-2**; **M-3** and **M-4**; **M-5** and **M-6**; **M-7** and **M-8**) were obtained that have the same chromatographic behaviors and identical data in LC/MS^n. So the findings could not be validated just by LC/MS^n data (Hoehleet al., 2006). In these cases, preparation of metabolites and further identification based on NMR data must be done. Of course, the direct isolation of the metabolites from urine, bile, or feces of humans or animals has difficulties, but it is the most reliable method for the identification of metabolites.

In summary, we have determined the definitive structures of nine phase 1 reductive metabolites of demethoxycurcumin by mass spectra and NMR spectroscopy. In the urine, the major reductive metabolites are the hexahydro-demethoxycurcumin and the methyl ether products of hexahydro-demethoxycurcumin. In the feces, the dehydroxy products of hexahydro and octahydro-demethoxycurcumin are predominant. These results are important for the understanding of demethoxycurcumin metabolism in rats and should provide information and reference for the further metabolic investigation of demethoxycurcumin in humans. Screening of the bioactivities of the novel metabolite is presently under study.

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Footnotes

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Figure Legends

Fig. 1. Chemical structure of demethoxycurcumin

Fig. 2. Structure of curcumin, demethoxycurcumin and bisdemethoxycurcumin

Fig. 3. Significant HMBC (H→C) correlations of **M-1** and **M-2**

Fig. 4. Structures of demethoxycurcumin metabolites in rat urine and feces and possible metabolic pathways for their production

Fig. 5. ESI-MS spectra of $[M-H]^-$ ion of **M-1** and **M-2** (A), **M-3** and **M-4** (B), **M-5** and **M-6** (C), **M-7** and **M-8** (D), **M-9** (E) and demethoxycurcumin (F)

Fig. 6. ^{13}C-NMR spectra of **M-1** and **M-2** (A), **M-3** and **M-4** (B), **M-5** and **M-6** (C), **M-7** and **M-8** (D)

Fig. 7. Partial HMBC spectrum of **M-1** and **M-2**

Fig. 8. Partial HMQC spectrum of **M-9**

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Table 1. *Assignments of carbon and proton signals of **M-1** and **M-3***

NO.	Carbon Signals		Proton Signals	
	M-1	M-3	M-1	M-3
1	29.5	31.7	2.80(2H, m)	2.60(1H, m); 2.70(1H, m)
2	44.6	39.4	2.67(2H, m)	1.72(2H, m)
3	211.2	71.4		3.62(1H, m)
4	42.9	37.4	2.39(2H, t, 6.8Hz)	1.49(2H, m)
5	23.2	25.2	1.57(2H, m)	1.35(1H, m); 1.47(1H, m)
6	31.1	31.8	1.51(2H, m)	1.60(2H, m)
7	34.7	34.9	2.50(2H, m)	2.54(2H, t, 7.6Hz)
1′	132.9	134.0		
2′	111.1	111.0	6.66(1H, d, 1.5Hz)	6.69(1H, br.s)
3′	146.4	146.4		
4′	143.8	143.7		
5′	114.3	114.3	6.81(1H, d, 8.0Hz)	6.83(1H, d, 7.8Hz)
6′	120.7	120.9	6.62(1H, brd, 8.0Hz)	6.67(1H, br.d, 7.8Hz)
1″	134.0	134.7		
2″, 6″	129.3(2C)	129.4(2C)	6.97(2H, d, 8.4Hz)	7.02(2H, d, 8.3Hz)
3″, 5″	115.1(2C)	115.1(2C)	6.74(2H, d, 8.4Hz)	6.74(2H, d, 8.3Hz)
4″	153.8	153.6		
-OCH_3	55.8	55.9	3.82(3H, S)	3.87(3H, s)

Notes: a) all spectra were recorded on an AV-600 spectrometer, in $CDCl_3$

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b) The carbon and proton signals were assigned unambiguously on ^{1}H NMR, ^{13}C NMR, HMQC and HMBC

c) m, multiplet.

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Table 2. *Assignments of carbon and proton signals of **M-2** and **M-4***

NO.	Carbon Signals		Proton Signals	
	M-2	M-4	M-2	M-4
1	28.9	31.1	2.80(2H, m)	2.60(1H, m); 2.70(1H, m)
2	44.5	39.3	2.67(2H, m)	1.72(2H, m)
3	211.2	71.3		3.62(1H, m)
4	42.9	37.4	2.39(2H, t, 6.8Hz)	1.49(1H, m)
5	23.3	25.2	1.57(2H, m)	1.35(1H, m); 1.47(1H, m)
6	31.1	31.8	1.51(2H, m)	1.60(2H, m)
7	35.3	35.6	2.50(2H, m)	2.54(2H, t, 7.6Hz)
1′	132.7	134.2		
2′, 6′	129.3(2C)	129.5(2C)	6.99(2H, d, 8.4Hz)	7.05(2H, d, 8.3Hz)
3′, 5′	115.3(2C)	115.2(2C)	6.74(2H, d, 8.4Hz)	6.75(2H, d, 8.3Hz)
4′	154.1	153.7		
1″	134.2	134.6		
2″	111.0	110.9	6.64(1H, d, 1.5Hz)	6.66(1H, br.s)
3″	146.3	146.3		
4″	143.5	143.5		
5″	114.1	114.1	6.81(1H, d, 8.0Hz)	6.82(1H, d, 7.7Hz)
6″	120.8	120.9	6.62(1H, br.d, 8.0Hz)	6.66(1H, br.d, 7.7Hz)
-OCH_3	55.8	55.9	3.84(3H, s)	3.87(3H, s)

Notes: a) all spectra were recorded on an AV-600 spectrometer, in $CDCl_3$

b) The carbon and proton signals were assigned unambiguously on ^{1}H NMR, ^{13}C NMR, HMQC

and HMBC

c) m, multiplet.

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Table 3. *Assignments of carbon and proton signals of **M-5** and **M-7***

NO.	Carbon Signals		Proton Signals	
	M-5	M-7	M-5	M-7
1	29.2	29.2	2.82(2H, m)	2.81(2H, m)
2	45.3	45.7	2.70(2H, m)	2.72(2H, m)
3	211.6	209.4		
4	49.3	47.3	2.53(2H, m)	2.69(1H, m); 2.43(1H, m)
5	66.9	76.7	4.03(1H, m)	3.71(1H, m)
6	38.2	35.8	1.62(1H, m); 1.76(1H, m)	1.75(2H, m)
7	30.7	30.4	2.59(1H, m); 2.71(1H, m)	2.59(2H, m)
1′	133.1	133.5		
2′	111.1	111.1	6.69(1H, br.s)	6.67(1H, br.s)
3′	146.4	146.4		
4′	143.9	144.0		
5′	114.4	114.3	6.81(1H, d, 7.9Hz)	6.81(1H, d, 7.9Hz)
6′	120.6	120.7	6.64(1H, br.d, 7.9Hz)	6.64(1H, br.d, 7.9Hz)
1″	132.5	132.8		
2″, 6″	129.4(2C)	129.3(2C)	7.01(2H, d, 8.1Hz)	6.99(2H, d, 8.6Hz)
3″, 5″	115.3(2C)	115.2(2C)	6.74(2H, d, 8.1Hz)	6.74(2H, d, 8.6Hz)
4″	154.1	154.0		
3’-OCH_3	55.9	55.8	3.85(3H, s)	3.83(3H, s)
5-OCH_3		56.9		3.31(3H, s)

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b) The carbon and proton signals were assigned unambiguously on ^{1}H NMR, ^{13}C NMR and HMQC.

c) m, multiplet.

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Table 4. *Assignments of carbon and proton signals of **M-6** and **M-8***

NO.	Carbon Signals		Proton Signals	
	M-6	M-8	M-6	M-8
1	28.7	28.6	2.80(2H, m)	2.80(2H, m)
2	45.3	45.6	2.70(2H, m)	2.72(2H, m)
3	211.6	209.4		
4	49.2	47.3	2.53(2H, m)	2.71(1H, m); 2.46(1H, m)
5	66.9	76.7	4.03(1H, m)	3.71(1H, m)
6	38.2	36.0	1.62(1H, m);1.76(1Hm)	1.75(2H, m)
7	31.4	31.0	2.59(1H, m); 2.71(1H, m)	2.60(2H, m)
1′	132.2	132.6		
2′, 6′	129.3(2C)	129.3(2C)	6.99(2H, d, 8.1Hz)	7.00(2H, d, 8.6Hz)
3′, 5′	115.4(2C)	115.3(2C)	6.74(2H, d, 8.1Hz)	6.73(2H, d, 8.6Hz)
4′	154.4	154.2		
1″	133.6	133.7		
2″	111.0	111.0	6.69(1H, br.s)	6.67(1H, br.s)
3″	146.4	146.4		
4″	143.6	143.6		
5″	114.3	114.3	6.81(1H, d, 7.9Hz)	6.82(1H, d, 8.0Hz)
6″	120.9	120.8	6.64(1H,br.d, 7.9Hz)	6.64(1H, br.d, 8.0Hz)
3″-OCH_3	55.8	55.8	3.85(3H, s)	3.85(3H, s)
5-OCH_3		57.0		3.31(3H, s)

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b) The carbon and proton signals were assigned unambiguously on ^{1}H NMR, ^{13}C NMR and HMQC.

c) m, multiplet.

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Table 5. *Assignments of carbon and proton signals of **M-9** and demethoxycurcumin*

NO.	Carbon Signals		Proton Signals	
	M-9	demethoxycurcumin	M-9	demethoxycurcumin
1	29.0	140.8	2.71(2H, t, 7.5Hz)	7.55(1H, d, *J*=16.2Hz)
2	41.6	120.9	2.84(2H, t, 7.5Hz)	6.77(1H, d, *J*=16.2Hz)
3	199.2	183.4		
4	127.7	101.0	6.22(1H, d, 15.5Hz)	6.05(1H, s)
5	143.6	183.3	7.35(1H, dd, 10.4, 15.4Hz)	
6	124.2	121.1	6.93(1H, dd, 10.2, 15.4Hz)	6.70(1H, d, *J*=15.8Hz)
7	141.9	140.5	6.96(1H, d, 16.0Hz)	7.54(1H, d, *J*=15.8Hz)
1′	131.4	125.9		
2′, 6′	129.2	130.5	7.00(2H, d, 8.1Hz)	7.57(2H, d, *J*=8.0Hz)
3′, 5′	115.1	116.0	6.64(2H, d, 8.1Hz)	6.82(2H, d, *J*=8.0Hz)
4′	155.5	159.9		
1″	128.1	126.4		
2″	110.3	111.3	7.16(1H, brs)	7,33(1H, brs)
3″	148.4	149.5		
4″	148.0	148.1		
5″	115.7	115.8	6.76(1H, d, 8.1Hz)	6.82(1H, d, *J*=8.0Hz)
6″	121.9	123.3	6.96(1H, brd, 8.1Hz)	7.15(1H, brd, *J*=8.0Hz)
$-OCH_3$	55.7	55.7	3.80(3H, s)	3.84(3H, s)

Notes: all spectra were recorded on an AV-600 or ARX-300 spectrometer, in DMSO-*d6*.

Fig. 1

DMD Fast Forward. Published on June 6, 2007 as DOI: 10.1124/dmd.107.015008

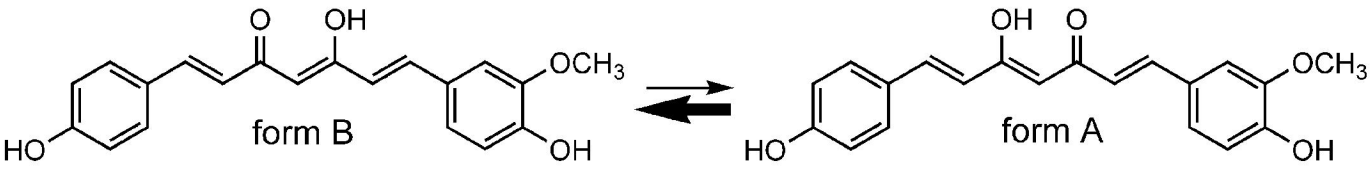
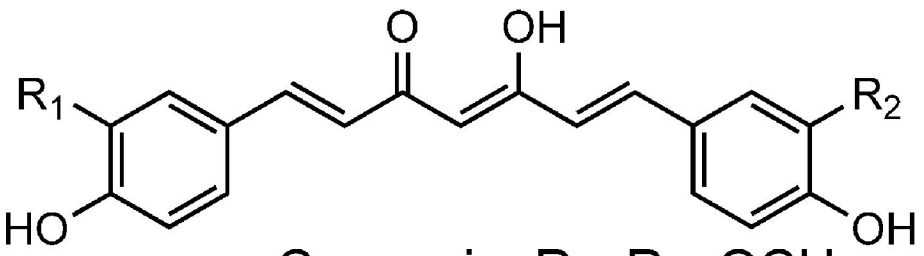


Fig. 2

DMD Fast Forward. Published on June 6, 2007 as DOI: 10.1124/dmd.107.015008



Curcumin: $R_1=R_2=OCH_3$

Demethoxycurcumin: $R_1=H$; $R_2=OCH_3$

Bisdemethoxycurcumin: $R_1=R_2=H$

Fig. 3

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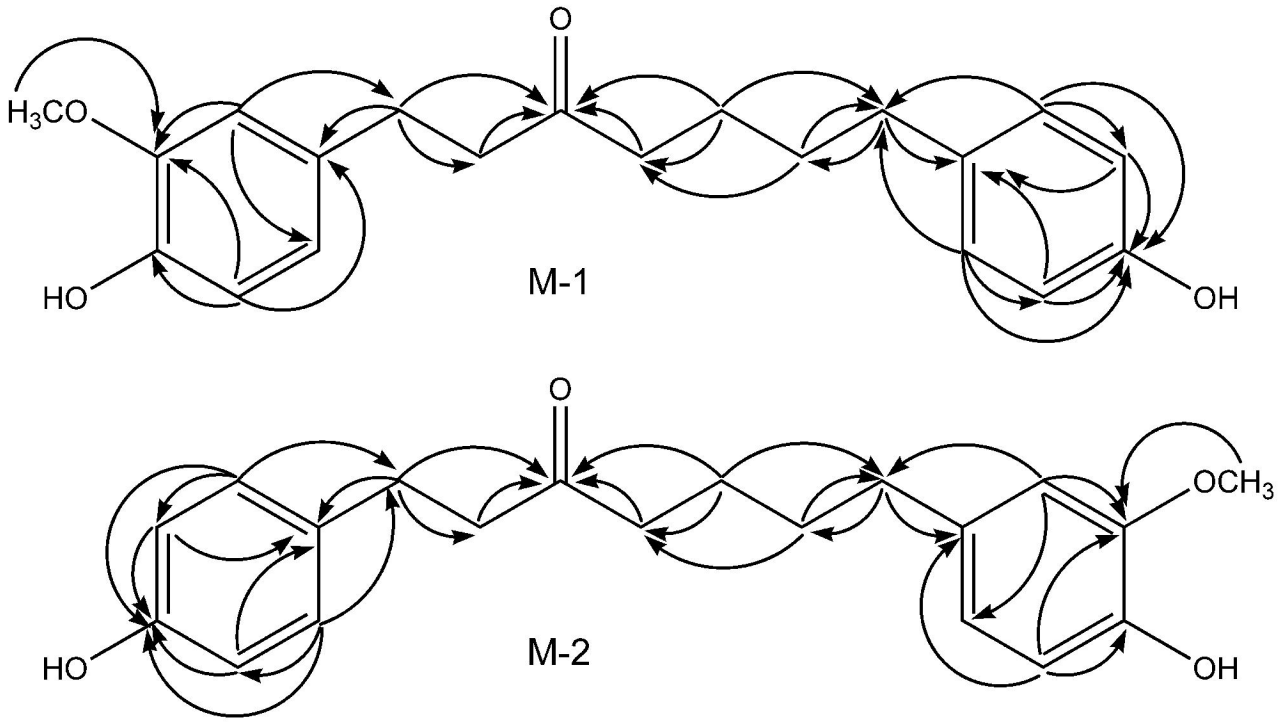

Fig. 4

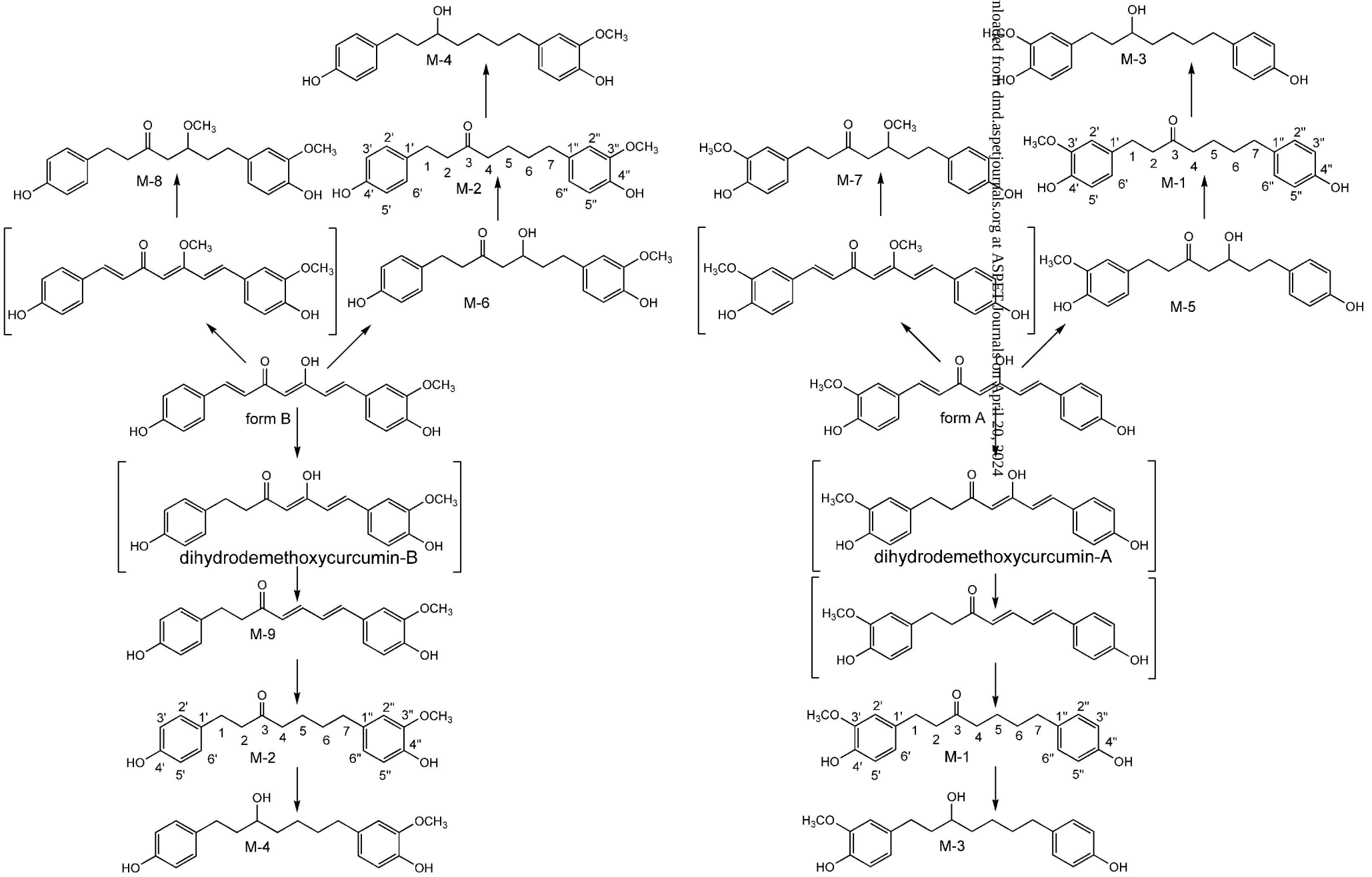

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Fig. 5

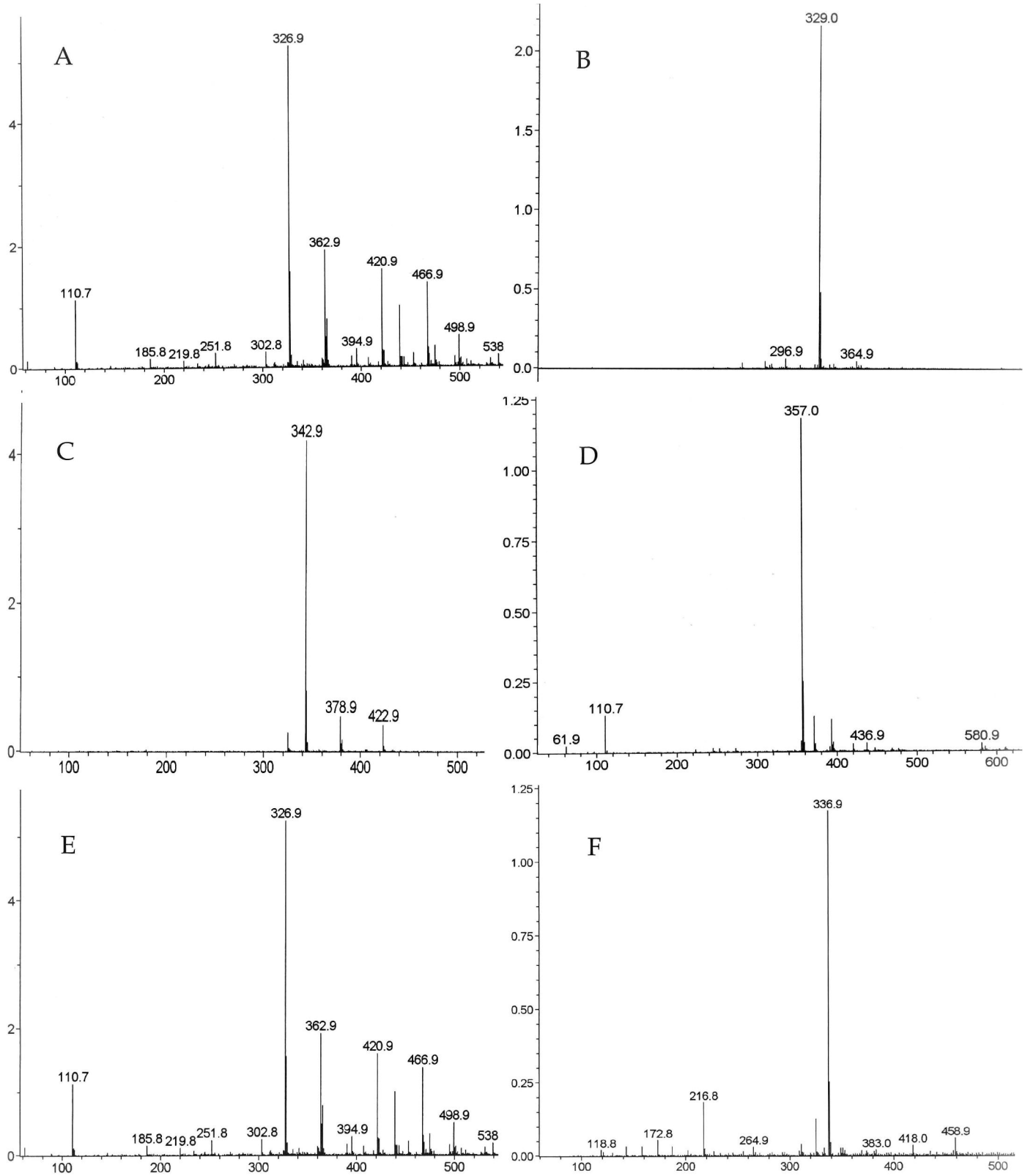

Fig. 6

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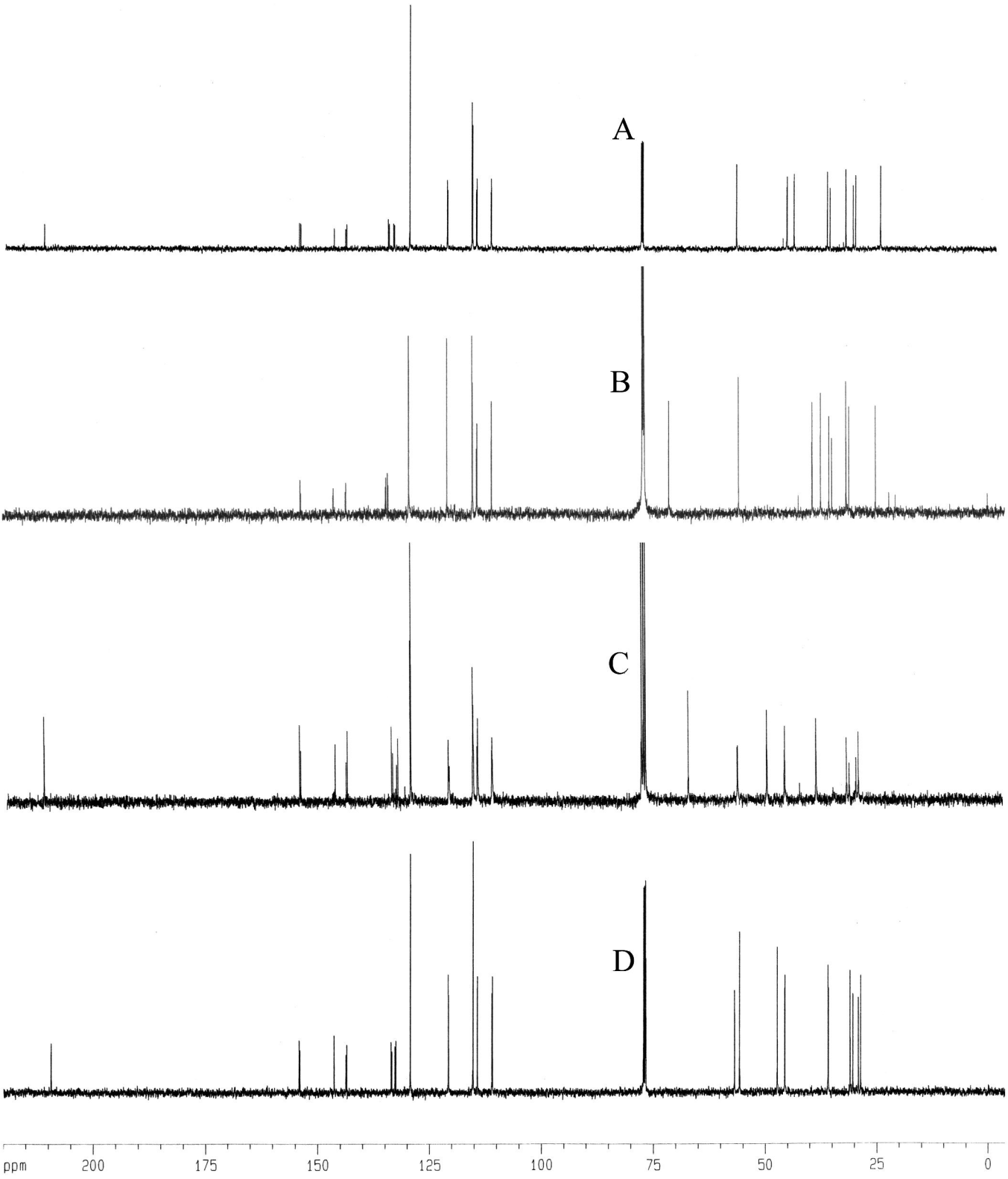

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Fig. 7

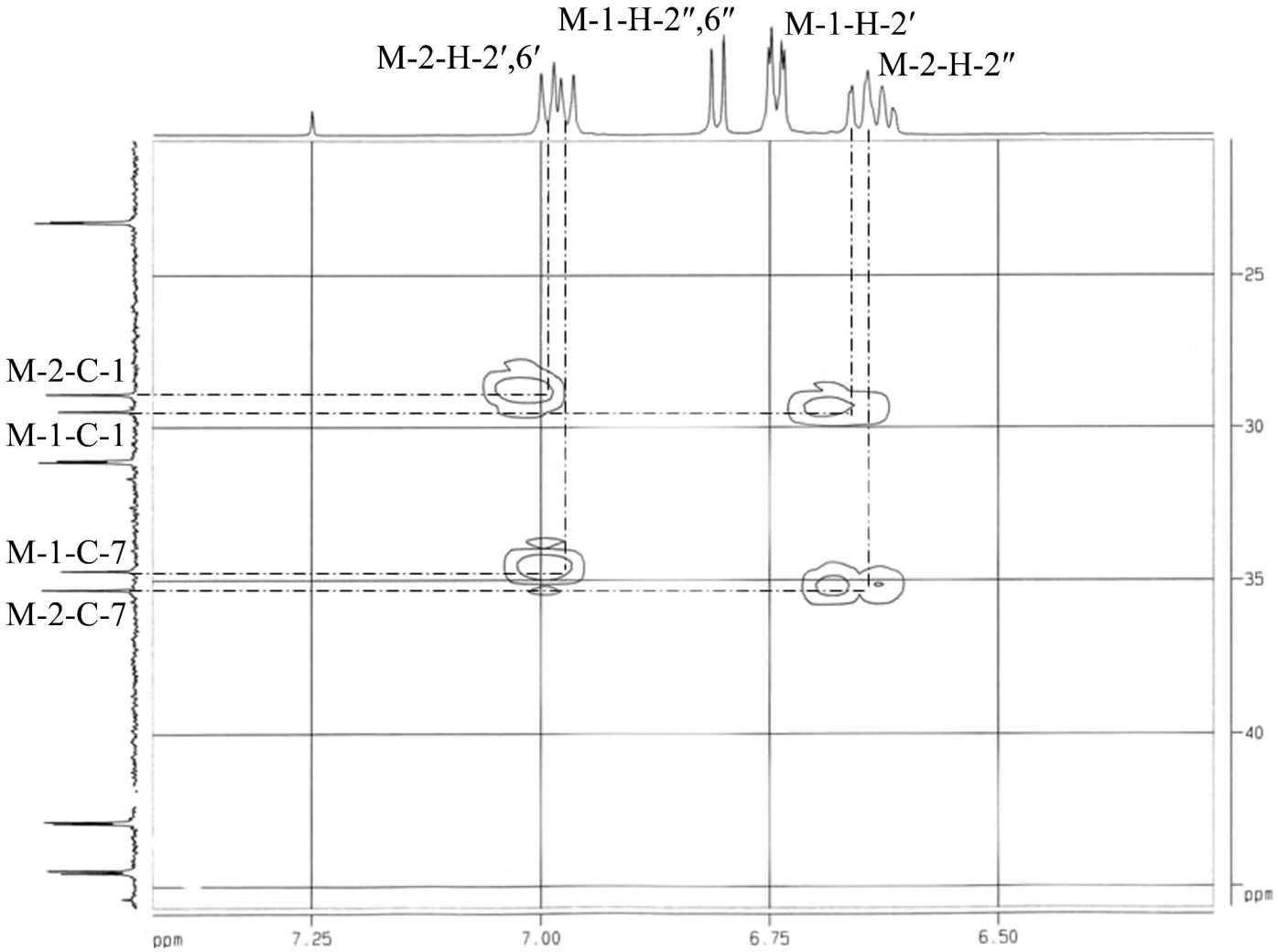

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Fig. 8