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SHORT COMMUNICATION

ANTI-AIDS AGENTS 65: INVESTIGATION OF THE *IN VITRO* OXIDATIVE METABOLISM OF 3',4'-DI-*O*-(-)-CAMPHANOYL-(+)-*CIS*-KHELLACTONE (DCK) DERIVATIVES AS POTENT ANTI-HIV AGENTS

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Running Title: Oxidative Metabolism of Anti-HIV DCK Analogs

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Abbreviations:

DCK, 3',4'-di-*O*-(-)-camphanoyl-(+)-*cis*-khellactone; HPLC, high performance liquid chromatography; SMR, structure-metabolism-relationship; ITMS, ion trap mass spectrometry; HIV, human immunodeficiency virus; CL_{int}, intrinsic clearance; $t_{1/2}$, half-life

Abstract

3',4'-Di-*O*-(-)-camphanoyl-(+)-*cis*-khellactone (DCK) is a synthetic khellactone ester that exhibits potent *in vitro* anti-HIV activity with a mechanism distinct from clinically used anti-HIV agents. Several series of mono- and di-substituted DCK derivatives (DCKs) have previously been synthesized, and their structure-activity-relationships are well established. In order to optimize DCK as a drug lead and to guide further structural modifications, metabolic stabilities and metabolite structures were analyzed. *In vitro* metabolic stabilities of DCKs in human liver microsomes were assessed using high performance liquid chromatography (HPLC) with UV detection to establish structure-metabolism relationships (SMR). HPLC coupled with ion trap mass spectrometry (ITMS) was used to identify the metabolite structures. The results indicated that DCKs undergo rapid oxidation on the lipophilic camphanoyl moieties and the substituents on the khellactone do not alter the rate or the metabolic pathways for this compound type. Our study suggested that the two camphanoyl ester moieties are the determinants of the low metabolic stability and that structural alteration in the two esters may be necessary to improve metabolic profiles of DCKs.

Introduction

3',4'-Di-*O*-S-(-)-camphanoyl-3'R,4'R-(+)-cis-khellactone, DCK, (**1**, Figure 1) is a natural product derivative with potent anti-HIV activity and a distinct mechanism of antiviral action (Huang et al., 1994). Over twenty derivatives of DCK (DCKs) have been synthesized to optimize the activity, some with EC_{50} values in the nanomolar range and high therapeutic indexes (Takeuchi et al., 1997;Xie et al., 1999;Xie et al., 2001;Xie et al., 2004). The DCK structure consists of a modified khellactone ring system and two camphanoyl ester moieties. Modifications of the khellactone ring greatly affect the *in vitro* activity, dependent both on the position and type of the substituents (Yu et al., 2003). Preliminary *in vivo* studies of several DCKs in rats indicated low oral bioavailability (0 -15%) and high clearance value close to the liver blood flow, corresponding to rapid *in vitro* metabolism by rat liver microsomes. Although absorption may also be an issue, the comparison of calculated log D values with the bioavailability indicated that it is not the only factor for the differences (Xie et al., 2004). These results prompted an evaluation of how the structure can influence metabolism of the DCKs. The study was based on the hypothesis that we can select or design a DCK analog with improved bioavailability by obtaining information about metabolic properties of the available DCKs. The objectives were to a) to investigate the metabolic stability of various DCKs in human liver microsomes using HPLC-UV, establish structure-metabolism-relationship (SMR) correlations and identify a possible structural motif and/or position that could provide improved stability, b) to determine the positions (3' or 4') of fragmentations of khellactone esters under MS/MS mode using structural analogs and c) to determine the sites of oxidative metabolism by analyzing metabolites using HPLC coupled with ion trap mass spectrometry (ITMS) and identify the structural motif that is metabolically vulnerable and subject to alteration.

Materials and Methods

Materials – DCK derivatives **1** – **14** (Figure 1), 4-methyl-*cis*-(+)-khellactone, and 3'-*O*–(*S*)-(-)-camphanoyl- 4'-*O*-adamantanecarbonyl-4- methyl-*cis*-(+)- khellactone (**15**, Figure 2) were previously synthesized in our laboratory. 3'-*O*-Adamantanecarbonyl-4'-*O*-(*S*)-(-)- camphanoyl-4-methyl-*cis*-(+)-khellactone (**16**, Figure 2) was synthesized from 4-methyl-*cis*-(+)-khellactone. NADPH, $MgCl_2$, acetic acid, dry pyridine, dry dichloromethane, camphanoyl chloride, and adamantanecarbonyl chloride were purchased from Sigma-Aldrich (St, Louis, MO). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburg, PA). Water for HPLC was obtained from distilled water, further purified in-house with a Millipore System (Billerica, MA). A pooled human liver microsome was purchased from Gentest (Woburn, MA). Waters Oasis Solid phase extraction (SPE) cartridges (3mL) were purchased from Waters Co. (Milford, MA).

Synthesis - 3'-*O*-Adamantanecarbonyl-4'-*O*-(*S*)-(-)-camphanoyl-4-methyl-*cis*-(+)-khellactone (**16**) was prepared by reacting 4-methyl-*cis*-kellactone and acyl chlorides sequentially in dry pyridine/dichloromethane (1:1, v/v, 1 mL) at room temperature overnight. 4-Methyl-*cis*-khellactone (276 mg, 1.0 mmol) was first reacted with camphanoyl chloride (432 mg, 2.0 mmol) to obtain 4'-*O*-(S)-(-)-camphanoyl-4'-hydroxy-4-methyl-*cis*-(+)-khellactone (160 mg, 35%), based on the procedure of Xie (Xie et al., 1999a). The isolated mono-ester (114 mg, 0.25 mmol) was then reacted with adamantanecarbonyl chloride (396 mg, 2.0 mmol) to obtain the final product (**16**, 11 mg, 7%). ^{1}H-NMR ($CDCl_3$) data are as follows: δ 1.03, 1.07, 1.08, (each 3H, s, CH_3 in camphanic acid), 1.44 and 1.46 (each 3H, s, 2'-CH_3 in khellactone), 1.71 and 1.93 (each 6H, m, CH_2 x6 in adamantane), 2.04 (3H, m, CH x3 in adamantane), 2.39 (3H, s, 4-CH_3 in khellactone), 1.66, 1.90, 2.61 and 2.52 (each 1H, m, 2 x CH_2 in camphanoyl group), 5.35 (1H, d,

J =4.8 Hz, 3'-H in khellactone), 6.10 (1H, s, 3-H in khellactone), 6.63 (1H, d, *J* =4.8 Hz, 4'-H in khellactone), 6.83 (1H, d, *J* =9.0 Hz, 6-H in khellactone), 7.51 (1H, d, *J* =9.0 Hz, 5-H in khellactone)

Sample Preparation – Stock solutions of DCKs (1 mg/mL) were prepared by dissolving the pure compound in acetonitrile and stored at 4ºC in the dark until use. For measurement of metabolic stability, DCKs (**1** – **14**) were brought to a final concentration of 1 μM with 100 mM sodium phosphate buffer at pH 7.4, which contained 0.1 mg/mL human liver microsome and 5 mM $MgCl_2$. Incubation volumes were 1.5 mL. Reactions were started by adding 150 μL of NADPH (final concentration of 1.5 mM) after 10 min incubation at 37ºC, and stopped by taking the aliquots over time, then adding to three volumes of ice-cold acetonitrile. Incubations of all samples were run in duplicate, and for control incubations, NADPH was omitted. For each sample, 200 μL aliquots were taken out at 0, 5, 10, 15, 30, and 60 min time points. After addition of acetonitrile and prior to centrifugation, fifteen μL of 0.01 mg/mL acetonitrile solution of 4-methyl-DCK (compound **3,** for incubation of **1**) or DCK (compound **1,** for incubations of **2**-**14**) was added to supernatants as an internal standard. The mixture was centrifuged at 15,000 rpm for 5 min. Supernatants were then evaporated to dryness under nitrogen using an N-Evap analytic evaporator (Organomation Associates Inc., Berlin, MA), and the residue was reconstituted with 150 μL of mobile phase (acetonitrile and water with 0.1% acetic acid, 1:1, v/v), then an aliquot (75 μL) was injected onto HPLC with UV detector. For LC-MS analysis of unknown metabolites, DCKs (**1** and **10**) were brought to a final concentration of 1 μM or 10 μM with 0.02 mg/mL or 0.2 mg/mL microsomal protein, respectively. Time dependent formation of metabolites was followed in 1 μM samples, and reactions were stopped at 0, 5, 10, 15, 30 and 60 min. To analyze fragmentation patterns of metabolite structures, the reaction was scaled up to 10 μM

concentration in 0.02 mg/mL microsomes, and stopped at 60 min without intermediate sampling points. Dried supernatants were evaporated, diluted with 50 µL acetonitrile, cleaned with an Oasis solid phase extraction (SPE) cartridge (Waters, Milford, MS), dried, reconstituted with 50 µL mobile phase (acetonitrile and water with 0.1% acetic acid, 3:7, v/v), and an aliquot (15 µL) was injected onto the LC-ITMS.

HPLC-UV Conditions – Analysis was carried out on a HP Series 1100 (Hewlett-Packard, Palo Alto, CA), consisting of an autosampler, degasser, binary pump, and variable wavelength UV detector. An Axxiom ODS column 150×4.6 mm I.D., 5 µm (Thomson Instrument Co., Springfield, VA) connected to a RP-18 guard column (15×3.2 mm I.D., 7 µm) (Brownlee, San Jose, CA) was isocratically eluted with acetonitrile and 25 mM acetic acid in water (55:45, v/v) at a flow rate of 1.5 ml/min. Detection wavelength was set at 320 nm, which was previously determined to be λ_{max} for the DCKs. Chromatograms were recorded on a Hewlett-Packard Chemstation A.05.01. (Hewlett-Packard, Palo Alto, CA). Standards for calibration graphs were prepared ranging from 0.020 to 100 µg/ml.

Calculations - In the determination of the *in vitro* half-life ($t_{1/2}$), the analyte/internal-standard peak height ratios were converted to percentage drug remaining, using the initial time (0 min) peak height ratio values as 100%. The slope of the linear regression from log percentage remaining versus incubation time relationships (-k) was used in the conversion to *in vitro* $t_{1/2}$; *in vitro* $t_{1/2}$ =0.693/k. Conversion to *in vitro* CL_{int} (in units of ml/min/mg protein) was done using the following formula (Obach et al., 1997) :

$$CL_{int} = (0.693/\ in\ vitro\ t_{1/2}) \times (\text{ml incubation /mg microsomes})$$

HPLC-MS Conditions – Analysis was carried out on a Surveyor LC system (Finnigan, San Jose, CA) interfaced to a LCQ Deca ion trap MS (Finnigan, San Jose, CA) with electrospray

ionization (ESI) source. A microbore Zorbax column SB-C18, 2.1 x 150 mm, 5 µm (Agilent, Wilmington, DE), was eluted with 0.1% acetic acid in water (A) and acetonitrile with 0.1% acetic acid (B) at a flow rate of 200 µL/min. Initially, B was held at 30% and increased linearly to 50% B at 5 min, then to 95% B at 23 min. The mobile phase was then returned to the initial condition and re-equilibrated for 7 min. The MS conditions were optimized with DCK to sheath gas at 50 arbitrary units (AU), auxiliary gas at 15 AU, capillary heated to 330°C, spray voltage at 5kV, tube lens offset at 25V. ESI was operated in the positive ion mode. Full scan spectra were acquired over the range of 180 to 800 *m/z*. Collision induced dissociation (CID) was applied to determine fragmentation of DCK **1** ($C_{34}H_{38}O_{11}$) and 3-chloro-4-methyl DCK **10** (Cl-Me-DCK, $C_{35}H_{39}ClO_{11}$) using 30 and 35% relative collision energy with isolation width (the width of the ion isolation band for the selected mass) of 1 and 7 (to observe Cl pattern in the fragment ion), respectively. For objective b, fragmentation of khellactone esters **3** ($C_{35}H_{40}O_{11}$), **15** ($C_{36}H_{42}O_9$), and **16** ($C_{36}H_{42}O_9$) were analyzed using direct infusion in a solution of acetonitrile/water (1:1, v/v) via a syringe pump at a flow rate of 10 µL/min.

Results

Metabolic Stabilities of DCKs in Human Liver Microsomes – *In vitro* metabolic stabilities of various DCK compounds were investigated using human liver microsomal preparations under oxidative conditions. The incubation mixtures of DCKs were analyzed by analytical HPLC with UV detection to measure the consumption of the parent compound. The structures of DCKs containing khellactone modifications and results of *in vitro* metabolic rates are shown (Figure 1). Introduction of substituents in the coumarin ring had no significant effect on the metabolic rates, regardless of the substituent type or the positions of the modification, except for 3-bromomethyl DCK (**12**). Compound **12** could not be detected even at time 0 min, due to the rapid conversion of the compound to 3-hydroxymethyl DCK (**13**). The hydrolysis of **12** to **13** was independent of the presence of NADPH or microsomes. The remaining DCKs were rapidly consumed in human liver microsomes with half-life values of the parent compound between 1.5 - 5.7 min.

Characterization of Khellactone Ester Fragmentation. The proposed fragmentation pattern and the observed mass fragment values of the khellactone esters **3**, **15** and **16** are shown (Figure 2). The product-ion spectrum of 4-methyl DCK (**3**, *m/z* 659, Na adduct) showed initial loss of a fragment corresponding to a camphanic acid from either the 3' or 4' position, which generates a chromene system (*m/z* 461) as a result of CID. Loss of CO_2 (*m/z* 615) was also observed, as is characteristic of many other coumarin compounds (Concannon et al., 2000). The remaining camphanoyl moiety could be further fragmented by subsequent CID, giving a product ion at *m/z* 303. Mass spectra of khellactone esters **15** and **16** allowed identification of the positions of fragment sources, which could not be achieved with compound **3**. These two compounds contained two different bulky acyl groups, camphanoyl on the 3'- and adamantanecarbonyl on

the 4'-position (**15**), or vice versa (**16**). In both cases, an abundant parent ion *m/z* 641, $[M+Na]^+$, was observed which dissociated to produce ions (*m/z* 461 for **15** and *m/z* 443 for **16**) attributable to the loss of the 4'-position acyloxy group. Subsequently, loss of the alkyl group from the remaining 3'-position ester generated ions at *m/z* 303, identical for the three khellactones. The patterns observed from 4-methyl DCK (**3**) and the two khellactone esters (**15** and **16**) were similar, therefore the same pattern is expected for other DCKs and also their possible metabolites, i.e. neutral loss of the 4'-position ester under MS/MS, followed by the sequential loss of alkyl from the 3'-position ester.

Characterization of DCKs Metabolites – The metabolism of DCKs **1** and **10** in human hepatic microsomes was investigated using positive mode with low (1 μM) and high (10 μM) substrate concentration incubations. At low concentration, a time dependent increase in multiple ion peaks corresponding to hydroxylated metabolites, $[M+16+Na]^+$, was observed. In order to identify the site of hydrdoxylation, the reaction was scaled up tenfold in concentration. Similarly to the low concentration incubates, the major metabolites were monohydroxylated. Representative extracted ion chromatograms of mono-hydroxylated metabolites of compound **1** and **10** (Figure 3B and 3D, respectively) showed multiple peaks that were not baseline resolved, and MS/MS and MS^3 were conducted for these mono-hydroxylated metabolites. Fragmentation patterns of the monohydroxy metabolites (Figure 4) suggested two types of metabolite strucutures: hydroxylation on the 4'-position camphanoyl (M2-M4 and M9-M10) or on the 3'- position camphanoyl (M1, M5, M6-M8, and M11). Hydroxylation on the khellactone moiety was not detected under the experimental conditions, corresponding to the SMR result showing that substituents on the coumarin did not impact the metabolic stability. Compound **10** has one Cl on the 3-position, and a Cl pattern was observed for both the parent and metabolites (when isolation width was set at 7),

which greatly aided confirmation of metabolite structures. No ion peaks corresponding to ester hydrolyzed khellactones, $[M-180+Na]^+$ or $[M-360+Na]^+$, were detected under the experimental conditions or in plasma (not shown). In addition, no ion peaks expected from general coumarin metabolism were observed: $[M-26+Na]^+$, $[M-24+Na]^+$, and $[M-10+Na]^+$, corresponding to *o*-hydroxyphenyl acetaldehyde, *o*-hydroxyphenylethanol, and *o*-hydroxyphenylpropionic acid, respectively. At higher concentration, multiple peaks corresponding to dihydroxylated $[M+32+Na]^+$, ketone $[M+14+Na]^+$, and hydroxy ketone/ carboxylic acid $[M+30+Na]^+$ metabolites were also observed. These metabolites were likely formed sequentially from mono-hydroxylated compounds.

Discussion

This is the first investigation on the metabolism of DCKs. In the present study using LC-UV, the rapid depletion of the parent compound did not allow for precision, however, the half-life values observed for DCKs (1.5 - 5.7 min) are shorter than reported values of highly cleared drugs despite the lower protein concentration (0.1 mg/mL) used. Obach reported *in vitro* half-life values between 8.0 – 11.0 min for the high clearance drugs propafenone, verapamil and diclofenac at microsomal protein concentration between 0.3 – 0.5 mg (Obach, 1999). Therefore, the rapid *in vitro* metabolic rates observed for the tested DCKs are predictive of high hepatic clearance *in vivo* and a high first pass effect. LC-UV chromatograms also indicated the production of multiple metabolites, regardless of the position or the type of the substituent on the khellactone (data not shown), and suggested similar metabolic pathways for various DCK derivatives. Subsequently, metabolites of selected DCK compounds were structurally

characterized using LC-ITMS. No hydrolysis of either camphanoyl ester moiety was observed under the hepatic microsomal incubation conditions employed. The absence of hydrolytic metabolism may be due to the steric hindrance of the bulky camphanoyl structure. Instead, hydroxylated metabolites were observed as primary oxidative metabolites in microsomal incubations, and were attributed mostly to hydroxylation of the two lipophilic camphanoyl moieties. This type of oxidation parallels that of camphor, a terpene that is structurally similar to camphanic acid, reported with rabbit P450 (White et al., 1984) and more commonly with prokaryotic $P450_{CAM}$ (Grogan et al., 2002), although we know of no report of human P450 oxidation of camphor.

In summary, we have demonstrated that DCK derivatives are rapidly and extensively metabolized by human liver microsomes under oxidative conditions. Although the substituents on the khellactone are reported to greatly affect the *in vitro* pharmacological activity, the results from both SMR and metabolite analysis indicate that these substituents are unlikely to influence the metabolic stability or the metabolic pathways of the compounds. The results suggest that incorporating alternative structures at the 3'- and/or 4'- ester position is the most viable approach to change the metabolic stability of these compounds.

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Footnotes

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Figure Legends

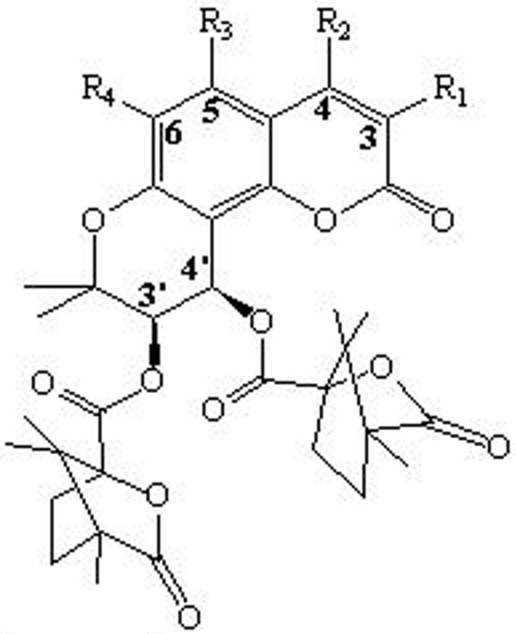
Figure 1. Chemical structures of DCK derivatives and Their *In Vitro* Metabolic Stabilities in Human Liver Microsomes.

Figure 2. Tandem mass spectra of khellactone esters; A) 4-methyl DCK B) 3'-*O*–(*S*)-(-)-camphanoyl-4'-*O*-adamantanecarbonyl-4-methyl-*cis*-khellactone and C) 3'-*O*-adamantanecarbonyl-4'-*O*-(*S*)-(-)-camphanoyl 4-methyl-*cis*-khellactone.

Figure 3. Extracted ion chromatograms of DCK (A), hydroxylated DCK metabolites (B), Cl-Me-DCK (C) and hydroxylated Cl-Me-DCK metabolites (D)

Figure 4. Tandem mass spectra fragments of the hydroxylated DCK metabolites M1-M5 (isolation width of 1 and CID=30) and the hydroxylated Cl-Me-DCK metabolites M6-M11 (isolation width of 7 and CID=35).

Figure 1



Compound
1: DCK.
3: 4-Methyl DCK.
10: Cl-Me DCK.
12: 3-Bromomethyl DCK.
13: 3-Hydroxymethyl 4-methyl DCK

compound	R_1	R_2	R_3	R_4	Halflife (min)	Intrinsic Clearance (ml/min/mg protein)
1	H	H	H	H	2.4	2.9
2	Me	H	H	H	5.1	1.4
3	H	Me	H	H	1.5	4.6
4	H	H	Me	H	2.1	3.3
5	H	H	H	Me	3.7	1.9
6	MeO	H	H	H	2.0	3.5
7	H	MeO	H	H	3.4	2.0
8	H	H	MeO	H	5.2	1.3
9	H	Me	Me	H	2.2	3.2
10	Cl	Me	H	H	2.0	3.5
11	H	Me	MeO	H	2.1	3.3
12	CH_2Br	Me	H	H	*	*
13	CH_2OH	Me	H	H	5.7	1.2
14	H	Me	H	CH_2OH	4.3	1.6

* : Parent compound could not be detected at time 0 min.

Figure 2

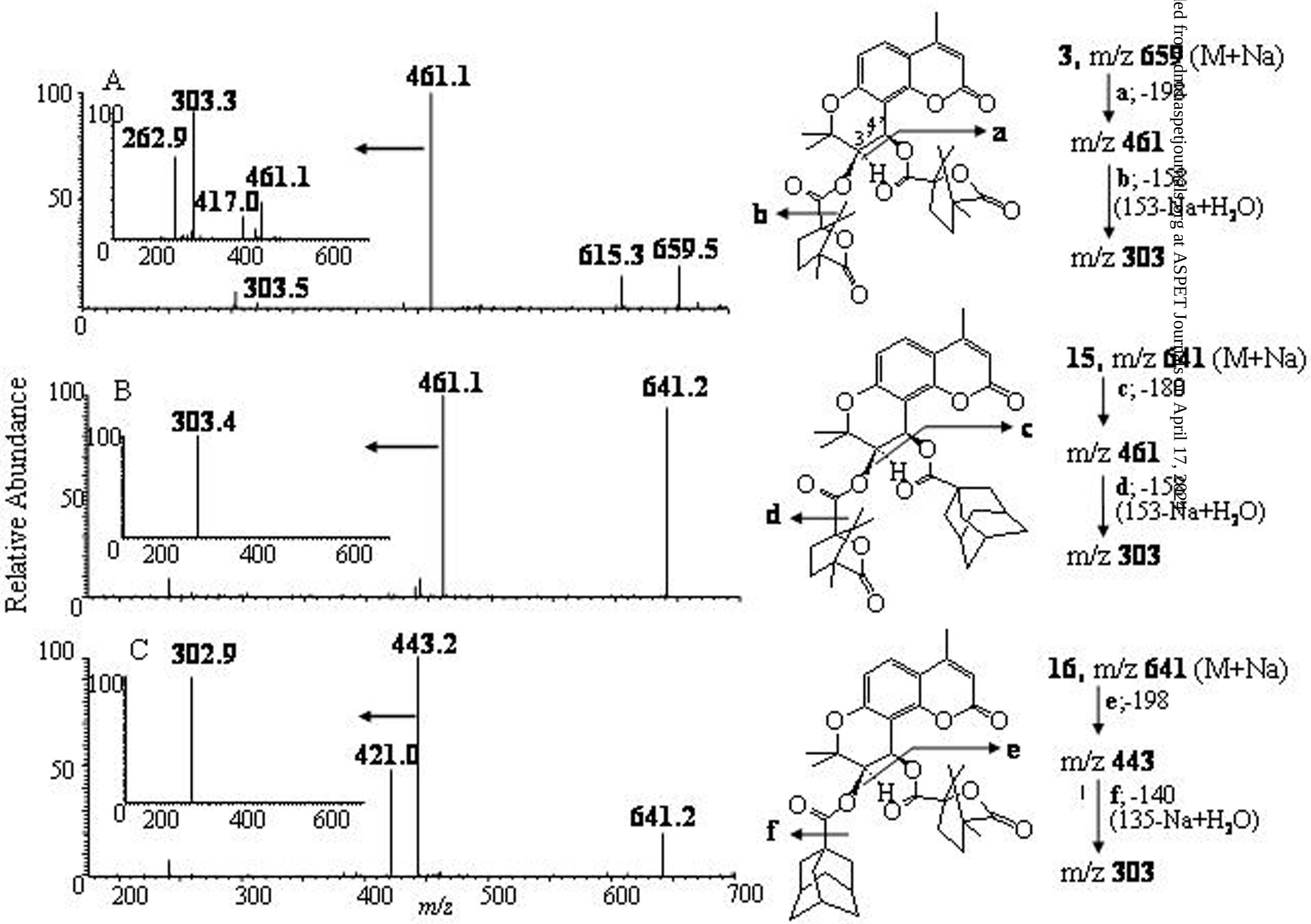


Figure 3

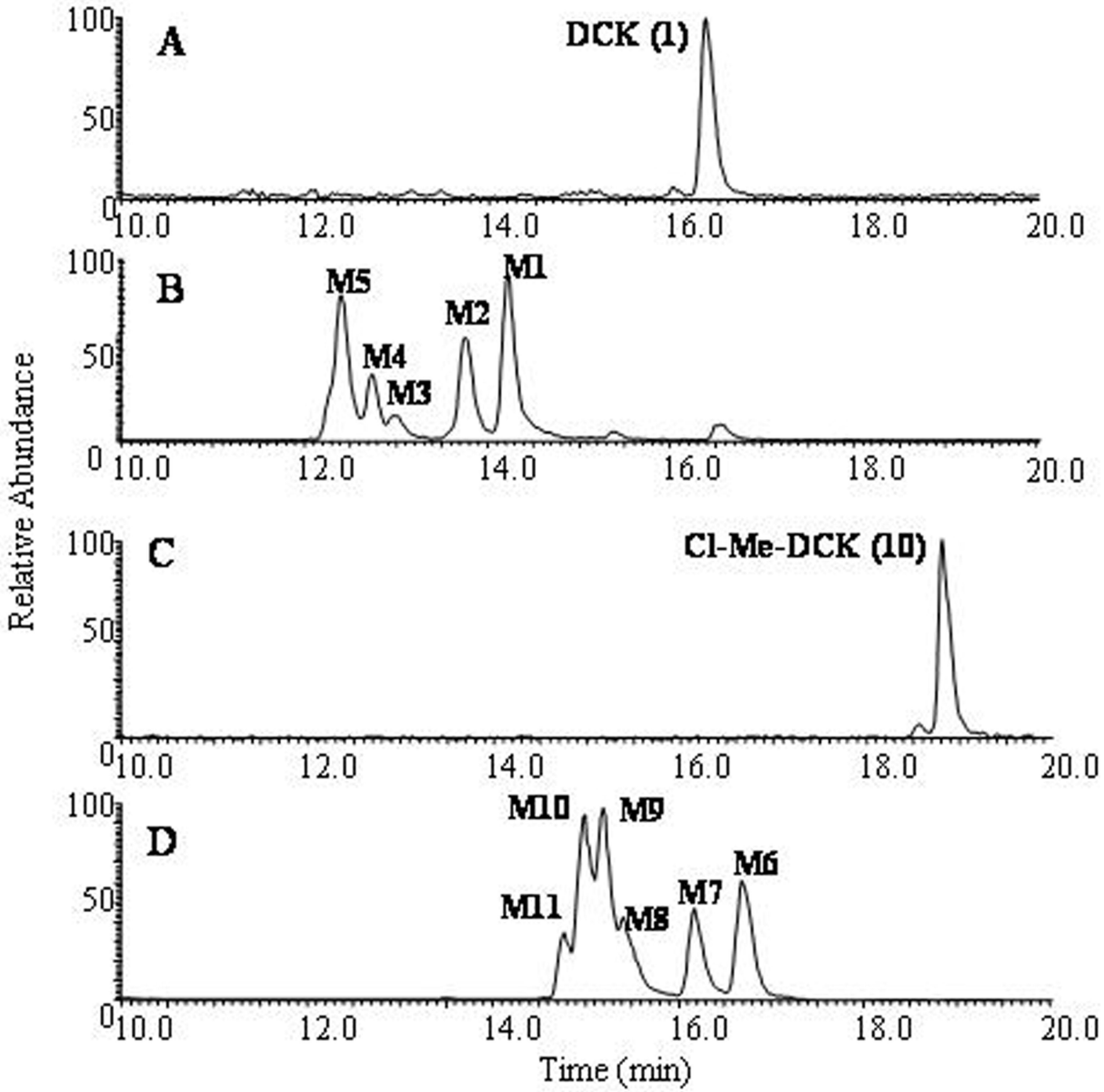
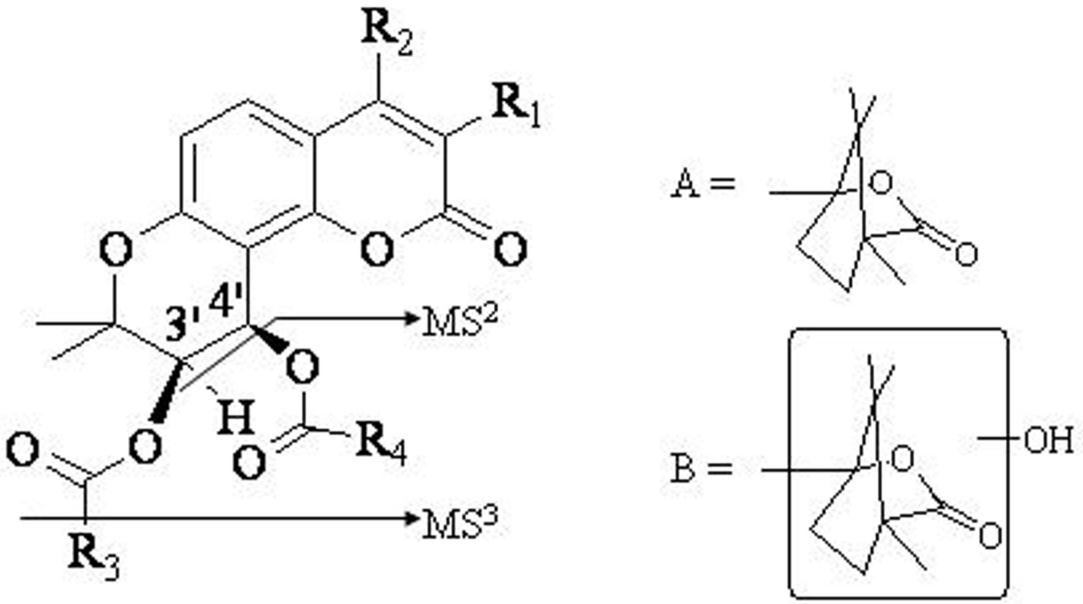


Figure 4



Peaks	Metabolite Structure				Mass Fragments (*m/z*)		
	R_1	R_2	R_3	R_4	MS^1	MS^2	MS^3
M2-M4	H	H	A	B	661	447	289
M1, M5	H	H	B	A	661	463	289
M9,M10	Cl	Me	A	B	709/711	495/497	337/339
M6-M8, M10, M11	Cl	Me	B	A	709/711	511/513	337/339