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## Highlights

- First quantitative study of fetomaternal transfer of CLO and its metabolites
- Highly accurate quantification using LC-MS/MS analysis
- Clear demonstration of the rapid passage of CLO through the placental barrier
- Metabolite-dependent differences observed in blood pharmacokinetics and residual levels

# Quantitative elucidation of maternal-to-fetal transfer of neonicotinoid pesticide clothianidin and its metabolites in mice

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**ABSTRACT**

Neonicotinoids (NNs), a widely used class of systemic pesticides, are regarded as exhibiting selective toxicity in insects. However, NNs are suspected of exerting adverse effects on mammals as well, including humans. To date, only adult male animal models have been subjected to general toxicity studies of NNs; fetuses have yet to be considered in this context. Here, we focused on the NN clothianidin (CLO) for the first quantitative LC-MS/MS analysis of maternal-to-fetal transfer and residual property of once-daily (single or multiple days), orally administered CLO and its metabolites in mice. The results revealed the presence of CLO and its five metabolites at approximately the same respective blood levels in both dams and fetuses. In the dams, CLO showed a peak value 1 h after administration, after which levels rapidly decreased at 3 and 6 h. In the fetuses of each group, levels of CLO were almost the same as those observed in the corresponding dams. The present results clearly demonstrated rapid passage of CLO through the placental barrier. However, metabolite-dependent differences observed in blood pharmacokinetics and residual levels. This is the first quantitative demonstration of the presence of CLO and its metabolites in fetal mouse blood.

**Key words:** clothianidin, maternal-to-fetal transfer, metabolites, mouse, neonicotinoid, quantitative LC-MS/MS

## 1. Introduction

The systemic pesticides collectively referred to as “neonicotinoids” (NNs) are chemically similar to nicotine. While NNs have been thought to exhibit low toxicity in birds and mammals, they are known to exert agonistic effects on the nicotinic acetylcholine receptors (nAChRs) of insects, affecting their central nervous system and leading to eventual paralysis and death (Ihara *et al*., 2003; Tomizawa and Casida, 2005). These systemic pesticides are taken up by, and transported throughout, plants, thereby protecting them from harmful insects for extended durations. In birds and mammals, the NNs have been shown to cause less toxicity than organophosphate and carbamate insecticides (Tomizawa and Casida, 2005). Vertebrates and insects have differently composed receptor subunits and receptor structures, which accounts for the higher affinity of NNs for the nAChRs of insects than for their counterpart vertebrate receptors (Latli *et al*., 1999; Tomizawa and Casida, 2005).

However, recent studies have reported reproductive toxicity in quails (Tokumoto *et al*., 2013; Hoshi *et al*., 2014) and adverse neurobehavioral effects in mice and rats (Hirano *et al*., 2015, 2018; Dhouib *et al*., 2017; Takada *et al*., 2018; Yoneda *et al*., 2018). In addition, it has been shown that exposure of perinatal mice to NNs causes abnormalities in germ cells (Yanai *et al*., 2017) and induction of anxiety-like behaviors (Tanaka , 2012; Sano *et al*., 2016) in their offspring. Such studies have suggested that NNs are almost certainly transferred from mother to fetus. Epidemiological investigations have also detected NNs in the urine of Japanese adults (Ueyama *et al*., 2015) as well as Japanese children (Ikenaka *et al*., 2019). It is therefore generally assumed that humans are indeed exposed to NNs on a daily basis. The residue standard of pesticides is set based on the acceptable daily intake (ADI) value calculated from the results of animal toxicity studies, in which almost exclusively adult individuals have been examined. Generally, fetuses are more sensitive to potentially toxic chemicals than are adults. Even if safe for adults, pesticides are not always equally safe for fetuses. At the very least, fetal safety studies require the collection of data regarding the extent to which pesticides are transferred from mother to fetus. Some chemicals have previously been reported in samples of umbilical cord blood (Todaka and

Mori, 2002), thereby demonstrating fetal exposure. However, no quantitative or time-dependent analyses of maternal-to-fetal transfer of NNs have been reported.

The potency and effectiveness of NNs are determined primarily by the structural features of the overall molecule (Tomizawa and Casida, 2005). The molecular structures change with metabolism, producing several different metabolites (Roberts and Hutson, 1999; Klein, 2001, 2003; Environmental Protection Agency, 2003). Due to such changes, certain metabolites exhibit reduced affinity for insect receptors and increased affinity for mammalian receptors (Casida, 2011). A desmethyl metabolite of the NN thiamethoxam causes single-cell necrosis and an increase in apoptosis in the mouse liver (Green *et al*., 2005). Such findings demonstrate the importance of including consideration of metabolites in investigations of NN toxicity.

For this study, we selected clothianidin (CLO), one of the NNs reported to cause neurobehavioral effects in mice (Hirano *et al*., 2015, 2018). We then performed a quantitative analysis of maternal-to-fetal transfer of CLO and its metabolites in mice.

## 2. Materials and methods

### *2.1. Experimental animals and procedure*

Male and female ICR mice (8-12 weeks old) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). All mice were maintained in individual (40.5 × 20.5 × 18.5 cm) ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) under controlled temperature (23 ± 2°C) and humidity (50 ± 10%) on a 14-h light/10-h dark cycle at the Kobe University Life-Science Laboratory with *ad libitum* access to a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and water. Female mice in proestrus were mated 1:1 with males overnight, and females that had a vaginal plug at 12:00 noon the following day were designated as being at embryonic day (E) 0.5. We administered CLO (purity: 95%, extracted from Dantotsu® Sumitomo Chemical Co., Tokyo, Japan; Hirano *et al*., 2015) or vehicle (0.5% carboxymethylcellulose, 10 mL/kg) to pregnant ICR mice by oral gavage, and the treatment group was divided into a single-dose administration group and a daily (4 or 9 days) single-dose administration group (Fig. 1). In all groups (n = 5-6 mice

in each group), the administration concentration was set to 65 mg/kg/day with reference to the no-observed-adverse-effect level (NOAEL) of 65.1 mg/kg from a 78-wk dietary carcinogenicity study in female mice (Food and Agriculture Organization of the United Nations, 2016; Uneme *et al*., 2006), and two to four fetuses were selected from each dam. The single-dose administration group was divided into 4 subgroups, and blood was collected from the dams (posterior vena cava) and the fetuses (heart) under anesthesia with isoflurane at 1, 3, and 6 h after CLO administration on E18.5, respectively, after which, the animals were euthanized (Fig. 1A). The daily single-dose administration groups received CLO once per day from E10.5 and E15.5 to E18.5, and blood was collected from the mice 6 h after the final administration of CLO by the method described above (Fig. 1B). The groups were designated by time of administration as CLO-1h, CLO-3h, CLO-6h, CLO4d-6h, CLO9d-6h. This study was approved by the Institutional Animal Care and Use Committee (Permission #26-05-07) and was carried out according to the Kobe University Animal Experimentation Regulations.

### *2.2. Chemicals*

The CLO standard (purity: 99.9%) was purchased from Fluka (Buchs, Switzerland). The CLO-d3 (purity: >97.0%) was purchased from Hayashi Junyaku (Osaka, Japan). The 1-methyl-3-nitroguanidine (MNG) (purity: >98.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Desmethyl-CLO (dm-CLO), desmethyl-desnitro-CLO (dm-dn-CLO), desnitro-CLO (dn-CLO) and CLO-urea were synthesized at Toho University (Supplement data).

### *2.3. Extraction of neonicotinoids from blood samples*

Here, 100 µl of 100-ppb CLO-d3 were placed in a 10-ml glass test tube as an internal standard substance. After the addition of 10 µl of blood and mixing the sample well, 0.5 ml of 1% formic acid in acetonitrile was added for protein precipitation. The tube contents were then briefly vortex-mixed. Then, 0.5 ml of acetonitrile and 0.5 ml of methanol were added to the tube, and the contents were each briefly vortex-mixed. The contents were

then sonicated for 10 min. Samples of supernatant were separated and used for the next extraction and purification step. Two types of solid-phase extraction cartridges, InertSep Phospholipid Remover (PR) (GL Science, Tokyo, Japan) and InertSep PSA (PSA) (GL Science), were connected in series (PR, top; PSA, bottom) and the samples were conditioned by the addition of 3 ml of acetonitrile. The samples were passed through the PR/PSA cartridges, and the eluents were collected in new test tubes (Fraction 1). After 0.5 ml of acetonitrile and 0.5 ml of methanol were sequentially passed through the PR/PSA cartridges, the PR was removed. Fraction 2 was also collected in the same test tube as that used for Fraction 1 (fraction 1+2). Furthermore, 3 ml of acetonitrile was passed through the PSA cartridge to elute all neonicotinoids, and the eluents were collected in the same test tube (Fraction 1+2+3). The collected eluents were evaporated to dryness using a centrifugal concentrator (CVE-200D with UT-2000, Eyela, Tokyo, Japan). The samples were then reconstituted with 200 µl of 20% methanol aqueous solution containing 100 ppb Cotinine-d3. Then they were transferred to a 1.5-ml tube and centrifuged at 10,000 G for 10 min. The supernatant was transferred into an HPLC vial for LC-MS/MS analysis.

An LC-ESI/MS/MS (Agilent 6495B, Agilent Co., CA, USA) system equipped with Kinetex Biphenyl (2.1 mm ID × 100 mm, ø 2.6 µm) (Phenomenex, Inc., CA , USA) was used for the sample analysis. Solvents A and B used for the HPLC analysis were 0.1% formic acid + 10 mM ammonium acetate water solution and 0.1% formic acid + 10 mM ammonium acetate methanol solution, respectively. The gradient was programmed as follows: $t$ = 0 to 1 min: 5% B (isocratic), $t$ = 6 min: 95% B (gradient), $t$ = 6 to 8 min (gradient): 95% B (isocratic). The column oven temperature and flow rate were 60ºC centigrade and 0.5 ml/min, respectively. Detection of target compounds was performed by multiple-reaction monitoring (MRM) in positive ionization mode as described in Table 1. The recovery rates of clothianidin and its metabolites were in the range of 62.1 ± 7.1% (MNG) to 92.3 ± 8.5% (dm-CLO). In addition, the reproducibility of the analysis system was confirmed by single or multiple analysts, with a relative standard deviation (RSD) of 10% for all compounds.

168

169 *2.4. Data analysis*

170 Statistical analyses were performed with Excel Statistics 2012 (Version 1.00, SSRI,
171 Tokyo, Japan). Levels of CLO and dm-CLO were analyzed by one-way ANOVA followed
172 by the Tukey-Kramer post hoc test. Levels of other chemicals were analyzed by Kruskal-
173 Wallis test followed by Steel-Dwass test. The results were considered significant when
174 the *p*-value was less than 0.05. The correlation between dams and fetuses regarding the
175 blood levels of CLO and dm-CLO were assessed using Pearson's correlation coefficient
176 analysis, and those of dm-dn-CLO, dn-CLO, CLO-urea, and MNG were done using
177 Spearman's rank correlation coefficient analysis.

178

179 **3. Results**

180 *3.1. Blood pharmacokinetics of CLO and its metabolites in dams and fetuses*

181 In the present study, CLO and its five metabolites were detected not only in dams but
182 also in the fetuses. There are several metabolic pathways for CLO. Here, we detected
183 desmethyl-CLO (dm-CLO), a major metabolite of CLO in mice that is generated by
184 demethylation of the methyl group of CLO. When dm-CLO is metabolized, the nitro
185 group is reduced to form desmethyl-desnitro-CLO (dm-dn-CLO). Thus, CLO forms not
186 only dm-CLO but also desnitro-CLO (dn-CLO) by reduction of the nitro group, and MNG
187 is formed from CLO by cleaving the thiazolyl chlorine substituent. Then, dn-CLO is
188 further metabolized to CLO-urea by cleavage of the imino group (Fig. 2).

189 Blood levels of CLO in both the dam and fetus groups were significantly higher in the
190 CLO-1h group (dams 23.69 ± 2.10 ppm, fetuses 20.46 ± 1.10 ppm) than in the CLO-3h
191 group (dams 16.19 ± 2.58 ppm, fetuses 14.48 ± 0.84 ppm), and blood levels in the CLO-
192 3h group were significantly higher than in the CLO-6h group (dams 7.29 ± 0.99 ppm,
193 fetuses 5.91 ± 0.43 ppm) (Fig. 3A). On the other hand, the blood levels of dm-CLO in the
194 CLO-3h group (dams 10.97 ± 1.01 ppm, fetuses 7.28 ± 0.32 ppm) and the CLO-6h group
195 (dams 10.27 ± 1.05 ppm, fetuses 6.50 ± 0.39 ppm) were significantly higher than in the
196 CLO-1h group (dams 5.53 ± 0.34 ppm, fetuses 2.90 ± 0.17 ppm) (Fig. 3B). The blood

197 levels of dm-dn-CLO in the CLO-6h group of dams (0.036 ± 0.002 ppm) were
198 significantly higher than those in the CLO-1h group of dams (0.022 ± 0.004 ppm);
199 however, in the fetuses there was no corresponding significant difference, although a
200 tendency was observed by which dm-dn-CLO levels increased with time (Fig. 3C). The
201 blood levels of fetal dn-CLO in the CLO-6h group (0.049 ± 0.004 ppm) were significantly
202 lower than those in the CLO-3h group (0.068 ± 0.005 ppm). Although the difference was
203 not statistically significant, dams in the CLO-6h group (0.050 ± 0.006 ppm) had lower
204 blood levels of dn-CLO than those in the CLO-3h group (0.061 ± 0.013 ppm) (Fig. 3D).
205 The blood levels of CLO-urea in both dams and fetuses gradually decreased over time. In
206 the dams, the blood levels of CLO-urea in the CLO-6h group (0.039 ± 0.004 ppm) were
207 significantly lower than those in the CLO-1h group (0.084 ± 0.009 ppm), and in the
208 fetuses, blood levels of CLO-urea in the CLO-6h group (0.039 ± 0.003 ppm) were
209 significantly lower than those in both the CLO-1h group (0.071 ± 0.005 ppm) and the
210 CLO-3h group (0.055 ± 0.003 ppm) (Fig. 3E). The blood levels of MNG were highest in
211 the CLO-3h group in both dams (0.307 ± 0.051 ppm) and fetuses (0.167 ± 0.010 ppm)
212 (Fig. 3F).
213
214 *3.2. Maternal-fetal ratio of levels of CLO and its metabolites in the blood*
215 There was a positive correlation between the blood levels of maternal CLO and its
216 metabolites and those of the offspring. The ratio of blood levels of each substance in the
217 fetus to those in the dam were 85.0% for CLO (Fig. 4A), 63.0% for dm-CLO (Fig. 4B),
218 84.5% for dm-dn-CLO (Fig. 4C), 96.7% for dn-CLO (Fig. 4D), 84.0% for CLO-urea (Fig.
219 4E), and 47.2% for MNG (Fig. 4F). For all compounds, blood levels in the fetus were
220 lower than those in the dams.
221
222 *3.3. Residual levels of CLO and its metabolites in the blood*
223 Since the transfer of CLO to the fetus was confirmed as described above, it was
224 administered daily for the purpose of confirming its residual property in both the dams
225 and fetuses. However, as regards maternal blood CLO levels, there was no significant

226 difference between the CLO-6h group (7.28 ± 0.99 ppm), the CLO4d-6h group (7.84 ±
227 0.74 ppm), and the CLO9d-6h group (6.67 ± 0.90 ppm). On the other hand, the blood
228 CLO level in the fetus was significantly higher in the CLO4d-6h group (6.57 ± 035 ppm)
229 than in the CLO9d-6h group (5.00 ± 0.31 ppm) (Fig. 5A). As regards dm-CLO, no
230 significant differences were observed between groups of dams, nor between groups of
231 fetuses (Fig. 5B). In the case of dm-dn-CLO, the blood level of dams was significantly
232 higher in the CLO9d-6h group (0.048 ± 0.004 ppm) than in the CLO4d-6h group (0.029
233 ± 0.004 ppm). As regards the corresponding fetal blood levels, those in the CLO9d-4h
234 group (0.033 ± 0.001 ppm) were significantly higher than those in both the CLO4d-6h
235 group (0.022 ± 0.001 ppm) and the CLO-6h group (0.029 ± 0.003 ppm), and those in the
236 CLO-6h group were significantly higher than in the CLO4d-6h group (Fig. 5C). In the
237 case of dn-CLO, maternal blood levels were significantly higher in both the CLO-6h
238 group (0.050 ± 0.006 ppm) and the CLO9d-6h group (0.053 ± 0.003 ppm) than in the
239 CLO4d-6h group (0.034 ± 0.002 ppm). Likewise, in the fetus, the CLO9d-6h group (0.046
240 ± 0.003 ppm) had significantly higher levels of dn-CLO than those in the CLO4d-6h
241 group (0.038 ± 0.003 ppm) (Fig. 5D). As regards CLO-urea, no statistically significant
242 differences were seen in maternal blood levels between groups, although the levels in the
243 CLO9d-6h group (0.030 ± 0.003 ppm) were lower than those in the CLO4d-6h group
244 (0.046 ± 0.009 ppm). As for CLO-urea in the fetal blood, the level in the CLO9d-6h group
245 (0.029 ± 0.002 ppm) was significantly lower than that of the CLO4d-6h group (0.036 ±
246 0.003 ppm) (Fig. 5E). Regarding MNG, the blood levels of the dams were not
247 significantly different between groups, but the CLO4d-6h group (0.144 ± 0.020 ppm) had
248 a higher level of MNG than that in the CLO9d-6h group (0.111 ± 0.012 ppm). On the
249 other hand, fetal blood levels of MNG were significantly higher in the CLO4d-6h group
250 (0.109 ± 0.006 ppm) than in the CLO9d-6h group (0.083 ± 0.005 ppm) (Fig. 5F).
251
252 **4. Discussion**

This study was the first to quantitatively confirm the transfer of CLO and its metabolites from dam to fetus in a mouse model. A time-dependent decrease in blood levels of CLO was observed in dams and fetuses in the single-dose administration group.

On the other hand, dm-CLO increased from 1 to 3 h after administration. Although the blood levels of dm-dn-CLO increased from 1 to 6 h, the levels of CLO-urea decreased with time. In the case of MNG, a peak was observed 3 h after the administration of CLO. From these findings, it was determined that the behavior of metabolites varies. This differential finding regarding the blood pharmacokinetics of CLO metabolites is thought to be due to differences between rapidly metabolized substances and those that are slower to metabolize. When the blood concentrations of maternal and fetal substances were compared, no substance-dependent differences were found to be associated with placental barrier permeability.

It is thought that variants of Cytochrome P450 (CYPs) are related to the metabolism of neonicotinoids (Shi *et al.*, 2009). Since most CYPs are negligibly expressed in fetal mice, it is expected that fetuses would not possess any capacity for CLO metabolism (Kitaoka *et al*., 2018). In fact, it is highly unlikely that a fetus could metabolize CLO transferred from the dam because the blood levels of CLO metabolites in the fetus were not higher than those of the dam. In addition, it was found that CLO crossed the placental barrier very quickly because it was transferred to the fetus at a high rate of 86% [CLO-1h group (fetus / maternal: 20.46 / 23.69 × 100)] of the maternal blood level 1 h after CLO administration. Furthermore, it was not only CLO that crossed the placental barrier quickly, but also dm-CLO at a rate of 63.0%, dm-dn-CLO at 84.5%, CLO-urea at 84.0%, and dn-CLO at 96.7%. These findings reveal that CLO and its metabolites were hardly inhibited by the placental barrier.

There were no significant differences observed in the blood CLO levels of the dams between the multiple days-of-exposure groups. In fetuses, the 4-day group showed significantly higher values than those of the 9-day group. From these findings, it can be concluded that the metabolism of the parent compound (CLO) is very fast and there is almost no residual property of CLO in the blood. On the other hand, dm-dn-CLO and dn-

CLO levels were lower in the 4-day administration group than in the 1-day administration group, and higher in the 9-day administration group than in the 4-day administration group, respectively. While it is thought that CLO exhibits little residual property, the same cannot be concluded about its metabolites. Of the five metabolites detected in this study, three substances (i.e., not dm-dn-CLO or dn-CLO) were found at lower levels or almost the same level in the 9-day group compared to the 4-day group. Thus, it will still be necessary to conduct experiments that include longer-term exposures.

Here, dn-CLO was detected in both dams and fetuses. Imidacloprid, a neonicotinoid, is metabolized to a desnitro form, thereby increasing affinity for mammalian target receptors (Casida, 2011). Similarly, dn-CLO has a higher affinity for mammalian target receptors than insects' ones (Casida, 2011). Taken together, these structural and metabolic findings contribute to the elucidation of the effects of CLO on mammals. However, it has been confirmed that the desnitro metabolic reaction is not active specifically in mice, since levels were several hundredths of those of dm-CLO.

Neurobehavioral effects and germ cell abnormalities in adult mice have been reported as effects of exposure to CLO (Hirano *et al*., 2015, 2018; Yanai *et al*., 2017). Likewise, mild focal necrosis with swollen cellular nuclei, hypertrophied blood vessels and cytoplasmic lesions in the mouse liver, and degeneration of the tubules and glomeruli in the kidney have been reported to be induced by imidacloprid (Arfat *et al*., 2014), as has suppression of humoral and cellular immune responses by thiamethoxam (Salema *et al*., 2016). The results of this study revealed that fetuses are also exposed to CLO at the same level as that in adults. Given that fetuses are thought to be more sensitive to chemicals than adults (Needham and Sexton, 2000; Charnley and Putzrath, 2001; Mori, 2004), the present findings raise concern about the potential for more serious adverse effects of CLO on fetuses. Neurobehavioral effects have been confirmed in male mice after the administration of CLO, even with the administration of one-tenth of the NOAEL dose (Hirano *et al*., 2018), which is close to the assumed acceptable daily intake (ADI); therefore, it is reasonable to extrapolate that CLO would also affect fetuses. The current NOAEL setting takes into account intake by adults only. Thus, the NOAEL values should

be recalculated to include non-adults, considering that fetuses would be exposed to toxins at the same levels as the dam. The ADI is calculated based on the NOAEL settings, and the residual standard value for food pesticide residues are set with reference to the ADI. Because there are NOAEL- and ADI-related concerns about effects on fetuses, it will be necessary to review the pesticide residual standard value of CLO.

In conclusion, the placental transfer of CLO was confirmed quantitatively for the first time in the present study. Specifically, this is the first report to quantitatively detect an NN, as well as its metabolites, in fetal blood. The fetuses are also potentially threatened by some metabolites which might have higher affinity for mammalian nAChRs than the parent compound (CLO). The present results thus provide important data for future elucidation of the cause of the effects of CLO on adults and fetuses.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.


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## References


Arfat, Y., Mahmood, N., Tahir, M.U., Rashid, M., Anjum, S., Zhao, F., Li, D.J., Sun, Y.L., Hu, L., Zhihao, C., Yin, C., Shang, P., Qian, A.R., 2014. Effect of imidacloprid on hepatotoxicity and nephrotoxicity in male albino mice. Toxicol. Rep. 1, 554–561.

Casida, J.E., 2011. Neonicotinoid metabolism: compounds, substituents, pathways, enzymes, organisms, and relevance. J. Agric. Food Chem. 59, 2918–2922.

Charnley, G., Putzrath, R.M., 2001. Children's health, susceptibility, and regulatory approaches to reducing risks from chemical carcinogens. Environ. Health Perspect. 109, 187–192.

Dhouib, I.B., Annabi, A., Doghri, R., Rejeb, I., Dallagi, Y., Bdiri, Y., Lasram, M.M., Elgaaied, A., Marrakchi, R., Fazaa, S., Gati, A., 2017. Neuroprotective effects of curcumin against acetamiprid-induced neurotoxicity and oxidative stress in the

developing male rat cerebellum: biochemical, histological, and behavioral changes. Environ. Sci. Pollut. Res. Int. 24, 27515–27524.

Environmental Protection Agency, 2003. Dinotefuran; notice of filing a pesticide petition to establish a tolerance for a certain pesticide chemical in or on food. Fed. Regist. 68, 39547–39554.

Food and Agriculture Organization of the United Nations, 2016. Food and Agriculture Organization of the United Nations FAO Specifications and Evaluations for Agricultural Pesticide Clothianidin (2016) (http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/Specs/Clothianidin2011.pdf)

Green, T., Toghill, A., Lee, R., Waechter, F., Weber, E., Noakes, J., 2005. Thiamethoxam induced mouse liver tumors and their relevance to humans. Part 1: mode of action studies in the mouse. Toxicol. Sci. 86, 36–47.

Hirano, T., Yanai, S., Omotehara, T., Hashimoto, R., Umemura, Y., Kubota, N., Minami, K., Nagahara, D., Matsuo, E., Aihara, Y., Shinohara, R., Furuyashiki, T., Mantani, Y., Yokoyama, T., Kitagawa, H., Hoshi, N., 2015. The combined effect of clothianidin and environmental stress on the behavioral and reproductive function in male mice. J. Vet. Med. Sci. 77, 1207–1215.

Hirano, T., Yanai, S., Takada, T., Yoneda, N., Omotehara, T., Kubota, N., Minami, K., Yamamoto, A., Mantani, Y., Yokoyama, T., Kitagawa, H., Hoshi, N., 2018. NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a novel environment. Toxicol. Lett. 282, 57–63.

Hoshi, N., Hirano, T., Omotehara, T., Tokumoto, J., Umemura, Y., Mantani, Y., Tanida, T., Warita, K., Tabuchi Y., Yokoyama, T., Kitagawa, H., 2014. Insight into the mechanism of reproductive dysfunction caused by neonicotinoid pesticides. Biol. Pharm. Bull. 37, 1439–1443.

Ihara, M., Matsuda, K., Otake, M., Kuwamura, M., Shimomura, M., Komai, K., Akamatsu, M., Raymond, V., Sattelle, D.B., 2003. Diverse actions of neonicotinoids on chicken α7, α4β2 and Drosophila–chicken SADβ2 and ALSβ2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. Neuropharmacology 45, 133–144.

Ikenaka, Y., Miyabara, Y., Ichise, T., Nakayama, S., Nimako, C., Ishizuka, M., Tohyama, C., 2019. Exposures of children to neonicotinoids in pine wilt disease control areas. Environ. Toxicol. Chem. 38, 71–79.

Kitaoka, S., Hatogai, J., Iimura, R., Yamamoto, Y., Oba, K., Nakai, M., Kusunoki, Y., Ochiai, W., Sugiyama, K., 2018. Relationship between low midazolam metabolism by cytochrome P450 3A in mice and the high incidence of birth defects. J. Toxicol. Sci. 43, 65–74.

Klein, O., 2001. Behaviour of thiacloprid (YRC 2894) in plants and animals. Pflanzenschutz-Nachr. Bayer 54, 209–240.

Klein, O., 2003. Behaviour of clothianidin (TI-435) in plants and animals. Pflanzenschutz-Nachr. Bayer 56, 75–101.

386 Latli, B.D., Amour, K., Casida, J.E., 1999. Novel and potent 6-chloro-3-pyridinyl ligands
387 for the α4β2 neuronal nicotinic acetylcholine receptor. J. Med. Chem. 42, 2227–
388 2234.
389 Mori, C., 2004. High-risk group and high-risk life stage: Key issues in adverse effects of
390 environmental agents on human health. Reprod. Med. Biol. 3, 51–58.
391 Needham, L.L., Sexton, K., 2000. Assessing children's exposure to hazardous
392 environmental chemicals: an overview of selected research challenges and
393 complexities. J. Expo. Anal. Environ. Epidemiol. 10, 611–629.
394 Roberts, T.R., Hutson, D.H.(Eds), 1999. Metabolic Pathways of Agrochemicals: Part 2:
395 Insecticides and Fungicides. Royal Society of Chemistry, U.K., p. 105–126 pp.
396 Salema, L.H., Alwan, M.J., Yousif, A.A., 2016. Immunotoxic effect of thiamethoxam in
397 immunized mice with *Brucella abortus* cultural filtrate antigen. Vet. World. 9, 1407–
398 1412.
399 Sano, K., Isobe, T., Yang, J., Win-Shwe, T.T., Yoshikane, M., Kawashima, T., Suzuki, G.,
400 Hashimoto, S., Nohara, K., Tohyama, C., Maekawa, F., 2016. In utero and lactational
401 exposure to acetamiprid induces abnormalities in socio-sexual and anxiety-related
402 behaviors of male mice. Front. Neurosci. 10, 228.
403 Shi, X., Dick, R.A., Ford, K.A., Casida, J.E., 2009. Enzymes and inhibitors in
404 neonicotinoid insecticide metabolism. J. Agric. Food. Chem. 57, 4861–4866.
405 Takada, T., Yoneda, N., Hirano, T., Yanai, S., Yamamoto, A., Mantani, Y., Yokoyama, T.,
406 Kitagawa, H., Tabuchi, Y., Hoshi, N., 2018. Verification of the causal relationship
407 between subchronic exposures to dinotefuran and depression-related phenotype in
408 juvenile mice. J. Vet. Med. Sci. 80, 720–724.
409 Tanaka, T., 2012. Effects of maternal clothianidin exposure on behavioral development
410 in $F_1$ generation mice. Toxicol. Ind. Health. 28, 697–707.
411 Todaka, E., Mori, C., 2002. Necessity to establish new risk assessment and risk
412 communication for human fetal exposure to multiple endocrine disruptors in Japan.
413 Congenit. Anom. 42, 87–93.
414 Tokumoto, J., Danjo, M., Kobayashi, Y., Kinoshita, K., Omotehara, T., Tatsumi, A.,
415 Hashiguchi, M., Sekijima, T., Kamisoyama, H., Yokoyama, T., Kitagawa, H., Hoshi
416 N., 2013. Effects of exposure to clothianidin on the reproductive system of male
417 quails. J. Vet. Med. Sci. 75, 755–760.
418 Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of
419 selective action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268.
420 Ueyama, J., Harada, K.H., Koizumi, A., Sugiura, Y., Kondo, T., Saito, I., Kamijima, M.,
421 2015. Temporal levels of urinary neonicotinoid and dialkylphosphate concentrations
422 in Japanese women between 1994 and 2011. Environ. Sci. Technol. 49, 14522–
423 14528
424 Uneme, H., Konobe, M., Akayama, A., Yokota, T., Mizuta, K., 2006. Discovery and de-
425 velopment of a novel insecticide 'clothianidin'. Sumitomo Kagaku 2, 1–14 (https://
426 www.sumitomo-chem.co.jp/english/rd/report/theses/docs/20060202_h6t.pdf).
427 Yanai, S., Hirano, T., Omotehara, T., Takada, T., Yoneda, N., Kubota, N., Yamamoto, A.,
428 Mantani, Y., Yokoyama, T., Kitagawa, H., Hoshi, N., 2017. Prenatal and early

postnatal NOAEL-dose clothianidin exposure leads to a reduction of germ cells in juvenile male mice. J. Vet. Med. Sci. 79, 1196–1203.

Yoneda, N., Takada, T., Hirano, T., Yanai, S., Yamamoto, A., Mantani, Y., Yokoyama, T., Kitagawa, H., Tabuchi, Y., Hoshi, N., 2018. Peripubertal exposure to the neonicotinoid pesticide dinotefuran affects dopaminergic neurons and causes hyperactivity in male mice. J. Vet. Med. Sci. 80, 634–637.

436 **FIGURE LEGENDS**

437 **Fig. 1. Outline of the experiments**

438 **(A)** Scheme showing the overall experimental design of a single-dose administration of
439 CLO. CLO was orally administered to pregnant ICR mice at embryonic day 18.5 (E18.5).
440 The single oral administration group was divided into three subgroups each of dams and
441 fetuses (total=six groups), from which blood samples were obtained at 1, 3, and 6 h after
442 CLO administration. The vehicle group was treated with 0.5% carboxymethyl-cellulose,
443 and blood samples were collected 1 h after the administration of vehicle, as in the CLO-
444 administration groups.

445 (B) Scheme showing the overall experimental design of a daily single-dose administration
446 of CLO. To examine CLO residual property, the CLO-6h group received a single dose at
447 E18.5, the CLO4d-6h group received four doses at E15.5-18.5, and the CLO9d-6h group
448 received nine doses at E10.5-18.5. Blood was collected in the same manner as described
449 in Fig. 1A after 6 h of the last CLO administration.

450

451 **Fig. 2.** Partial metabolic pathway of CLO in mice

452 Demethylation of the CLO methyl group forms dm-CLO, and dm-dn-CLO is produced
453 by further reduction of the dm-CLO nitro group. Reduction of the nitro group of CLO
454 produces dn-CLO, which in turn cleaves the imino group of dn-CLO to produce CLO-
455 urea. Metabolic cleavage of the thiazolyl choline group of CLO produces the substance
456 1-methyl-3-nitroguanidine (MNG).

457

458 **Fig. 3.** Blood pharmacokinetics of CLO and its metabolites in dams and fetuses

459 The parent compound (CLO) and its metabolites detected in maternal and fetal blood
460 after a single CLO administration are shown. The blood pharmacokinetics showed
461 various behaviors for each substance, but the dynamics were similar between the dams
462 and fetuses. Values are mean ± SE. **P*<0.05. The number of samples: CLO-1h (dam = 6,
463 fetus = 24), CLO-3h (dam = 6, fetus = 22), CLO-6h (dam = 6, fetus = 23)

464

**Fig. 4.** Maternal-fetal ratio of blood levels of CLO and its metabolites

The fetus-to-mother ratio of blood levels of CLO and its metabolites in the single-dose group. The horizontal axis (coordinates) shows the blood concentrations of the mothers and the vertical axis (coordinates) shows the blood concentrations of the offspring. The number of samples: CLO-1h (dam = 6, fetus = 24), CLO-3h (dam = 6, fetus = 22), CLO-6h (dam = 6, fetus = 23)

**Fig. 5.** Residual levels of CLO and its metabolites in the blood of dams and fetuses

Residual levels of the parent compound (CLO) and its metabolites detected in maternal and fetal blood after a daily single-dose CLO administration are shown. The blood pharmacokinetics showed various behaviors for each substance (A-F), but the general dynamics were similar between the dams and fetuses. Values are mean ± SE. **P*<0.05. The number of samples: CLO-6h (dam = 6, fetus = 23), CLO4d-6h (dam= 5, fetus = 20), CLO9d-6h (dam = 7, fetus = 28)

Table 1: Multiple-reaction monitoring (MRM), retention times (RT), recovery rate, and limit of quantification (LOQ) of target chemicals

| **Chemicals** | **MRM** | **Qualifier ion** | **RT** | **Recovery rate (%±SD)** | **LOQ (ng/ml)** |
|---|---|---|---|---|---|
| Clothianidin | 250.02>132.00 | 169.1 | 3.9 | 86.3 ±6.4 | 0.5 |
| Clothianidin-d3 | 253.04>132.10 | 171.9 | 3.9 | 86.8 ±7.2 | – |
| dm-Clothianidin | 236.00>132.00 | 113.1 | 2.9 | 92.3 ±8.5 | 1.0 |
| dm-Clothianidin-urea | 192.00>132.10 | 86.1 | 1.8 | 77.1 ±7.6 | 5.0 |
| Clothianidin-urea | 206.02>86.2 | 175.0 | 2.6 | 82.5 ±11.0 | 0.5 |
| dn-Clothianidin | 205.03>132.1 | 45.1 | 1.3 | 72.2 ±6.3 | 0.5 |
| dm-dn-Clothianidin | 191.02>132.1 | 45.2 | 1.2 | 74.4 ±7.2 | 1.0 |
| MNG | 119.10>73.02 | 57.2 | 1.0 | 62.1 ±7.1 | 0.5 |

Fig. 1

A: Scheme showing the overall experimental design of a single-dose administration of CLO

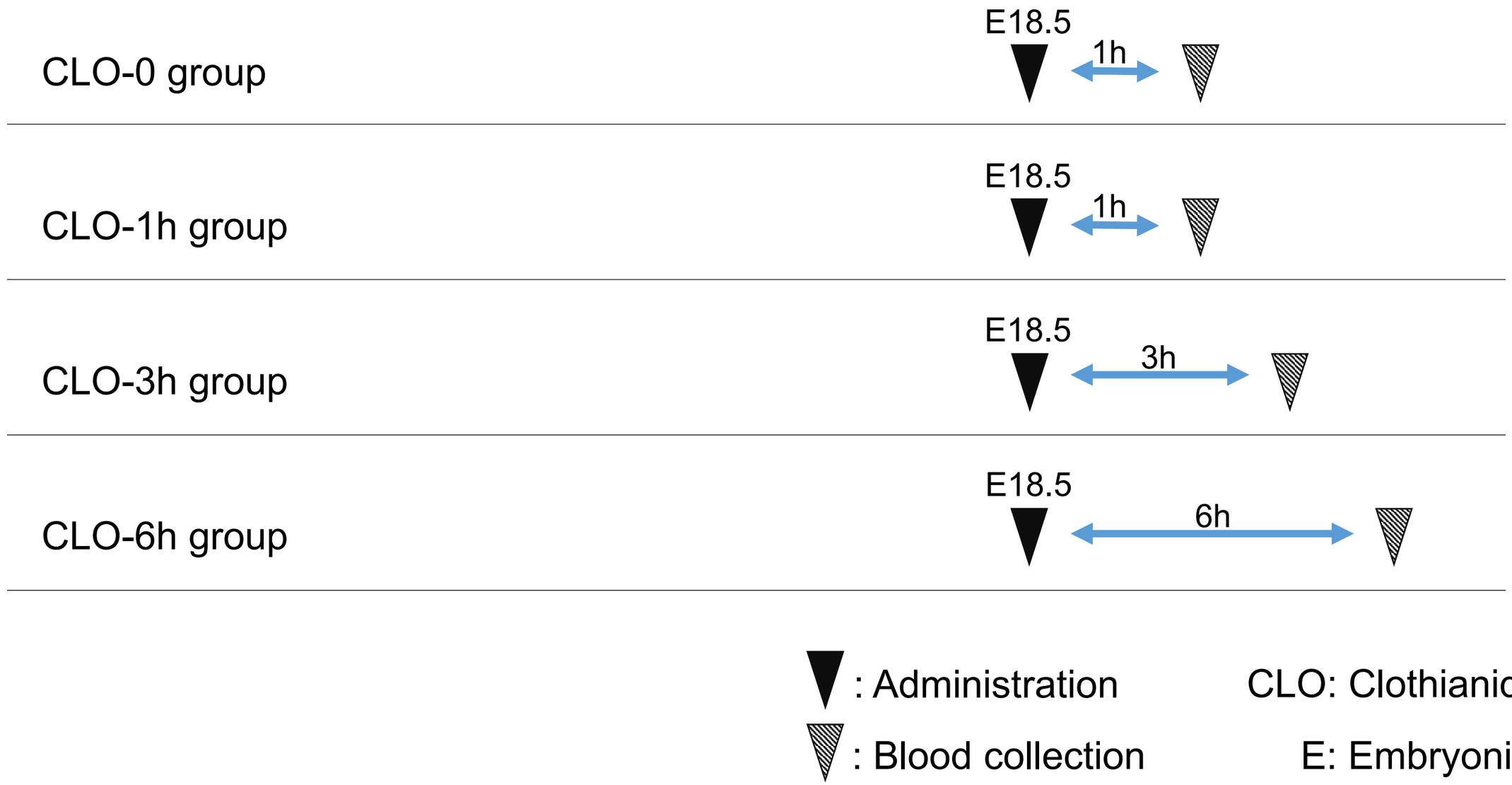


Fig. 1

B: Scheme showing the overall experimental design of a daily single-dose administration of CLO

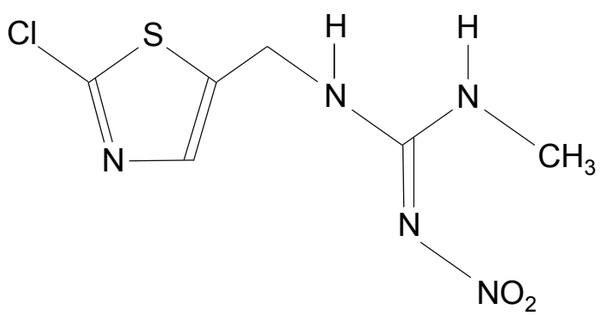

: Administration

: Blood collection

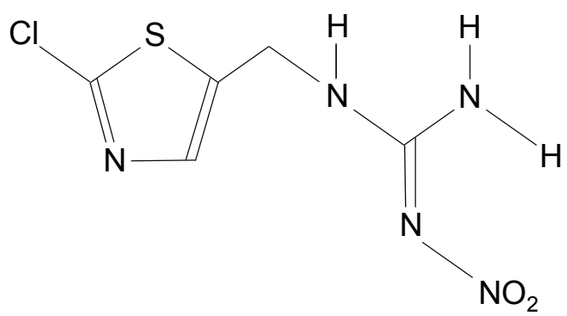
CLO: Clothianidin

E: Embryonic day

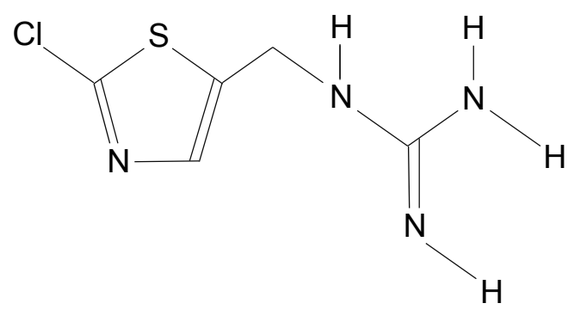
Fig. 2 Partial metabolic pathway of CLO in mice



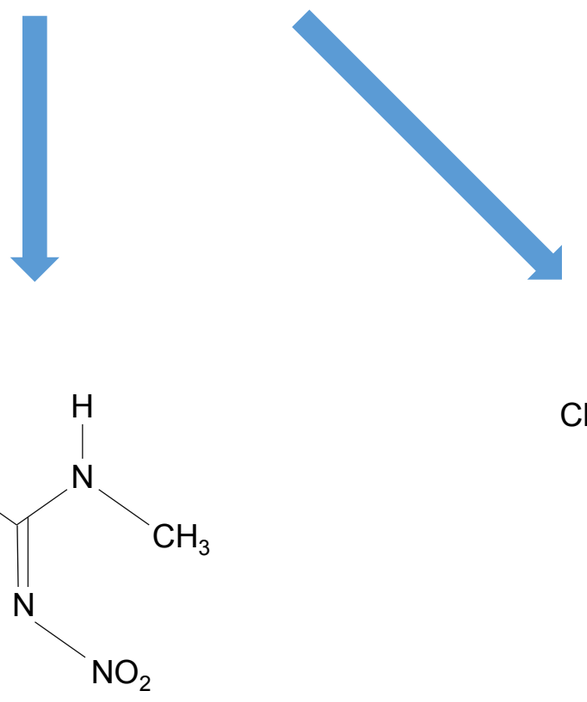

Clothianidin (CLO)



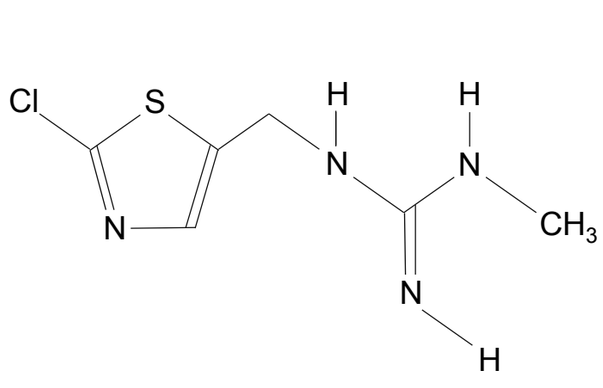

desmethyl-CLO (dm-CLO)



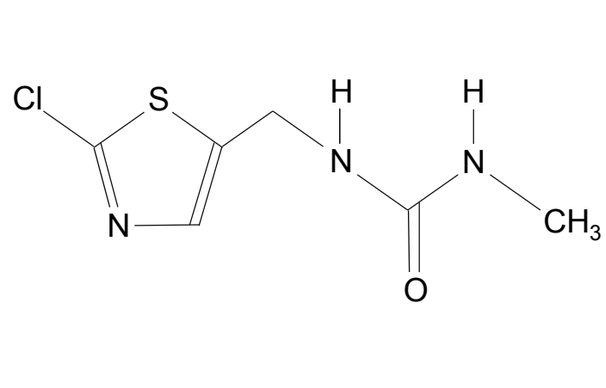

desmethyl-desnitro-CLO (dm-dn-CLO)




1-methyl-3-nitroguanidine (MNG)




desnitro-CLO (dn-CLO)




CLO-urea

Fig. 3 Blood pharmacokinetics of CLO and its metabolites in dams and fetuses

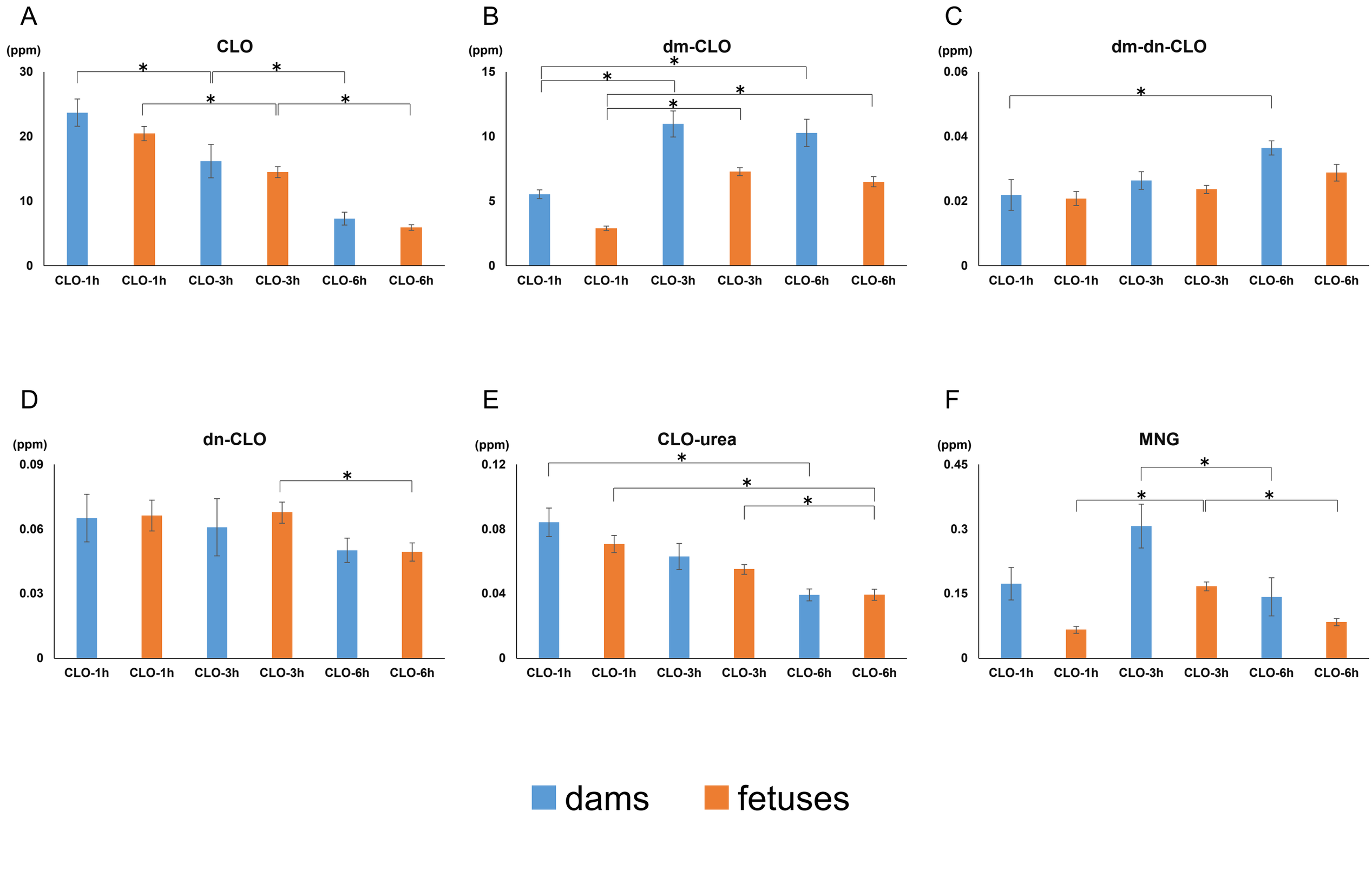


Fig. 4　Maternal-fetal ratio of blood levels of CLO and its metabolites

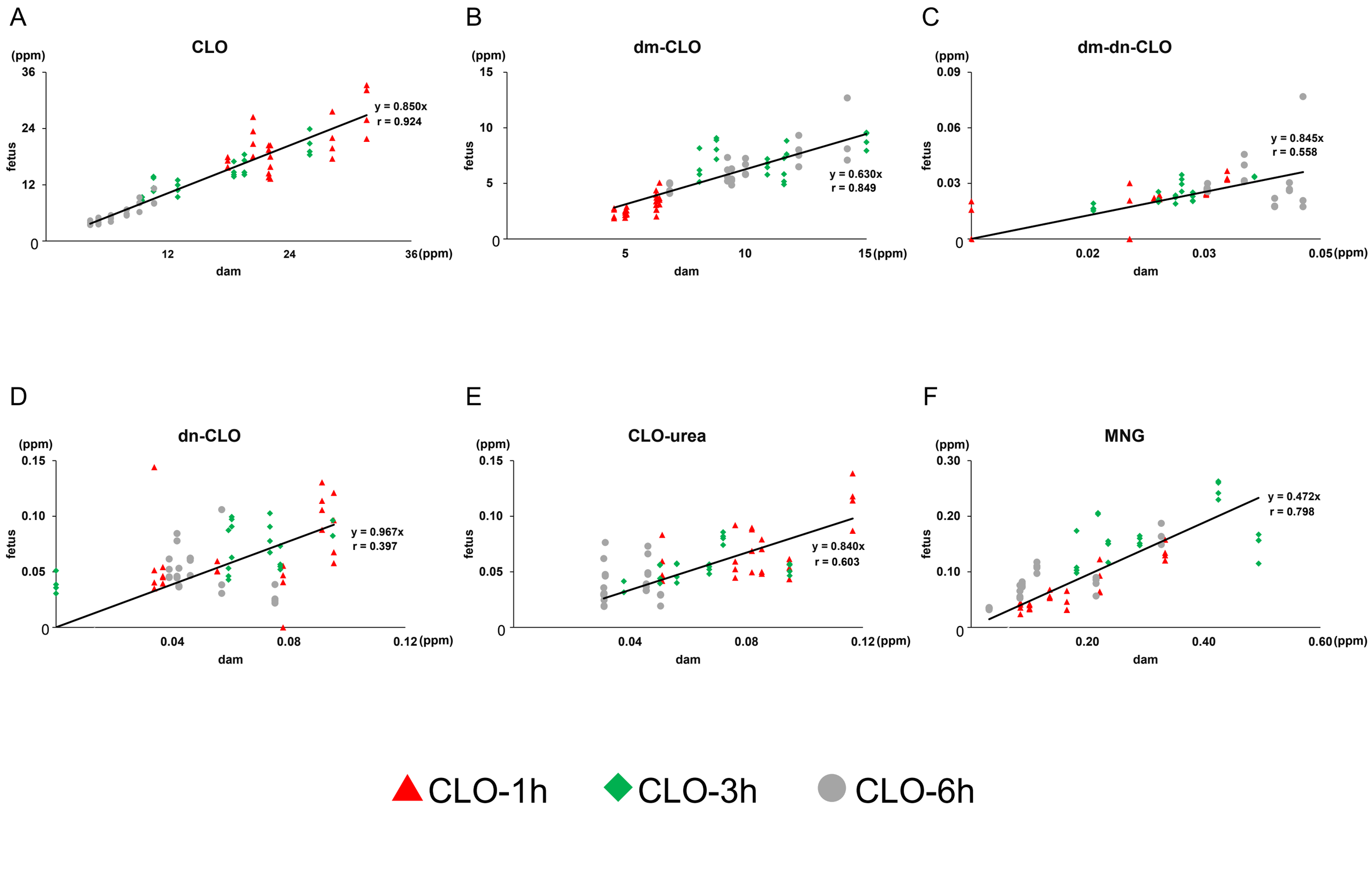
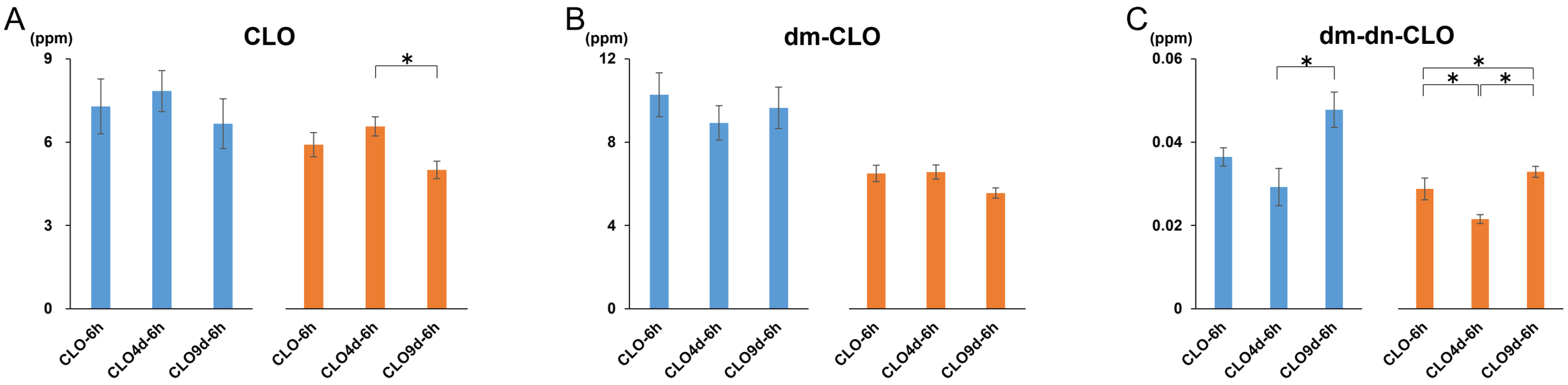
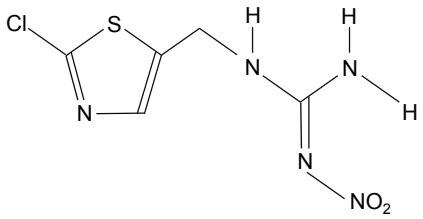


Fig. 5 Residual levels of CLO and its metabolites in the blood of dams and fetuses



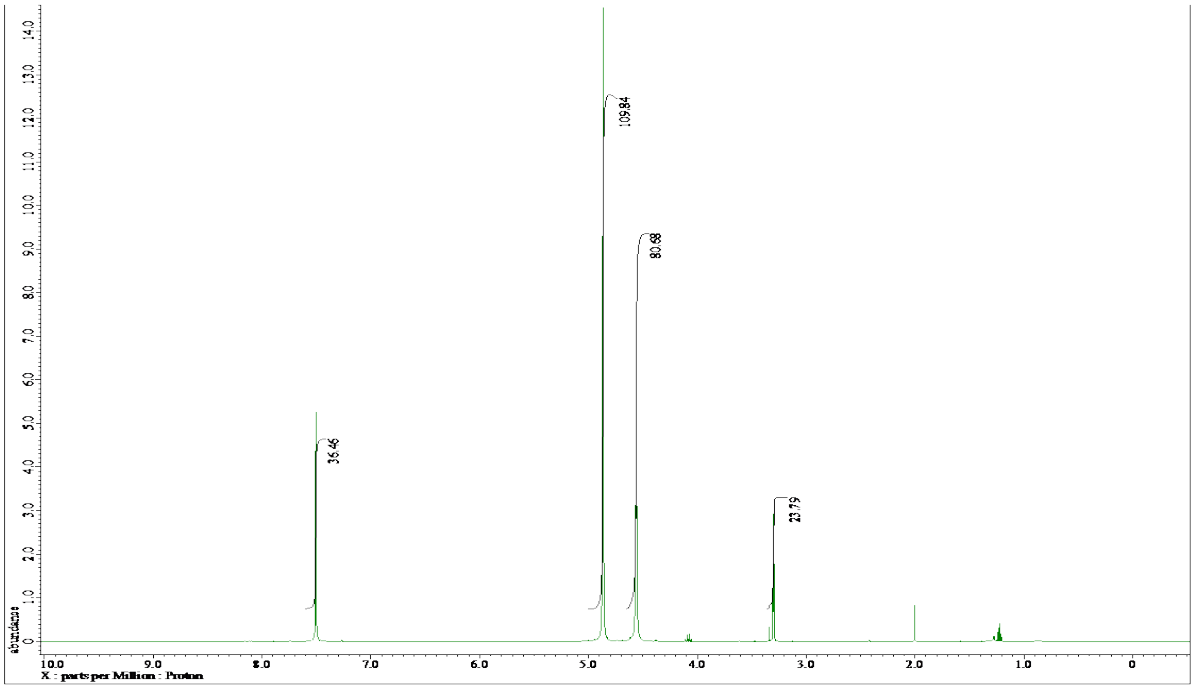
**Supplementary**

**Click here to download Supplementary: Suppl_Fig_Ohno_S_R2.pdf**

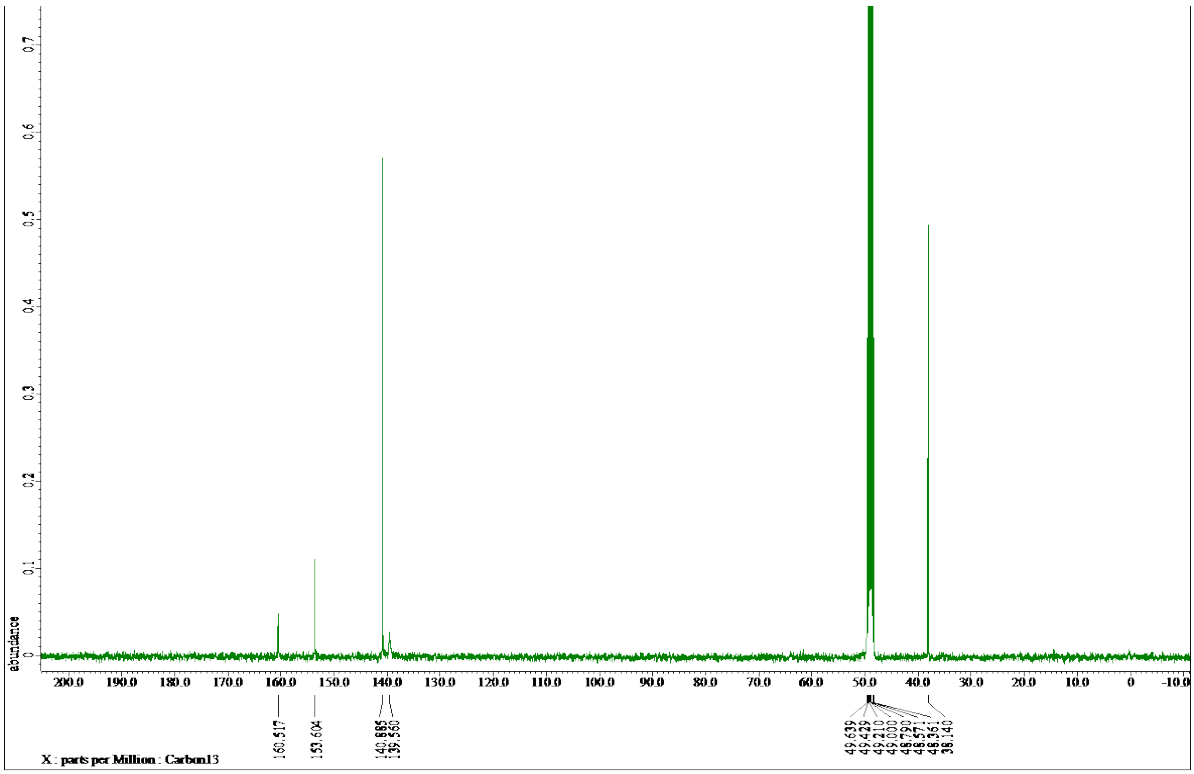


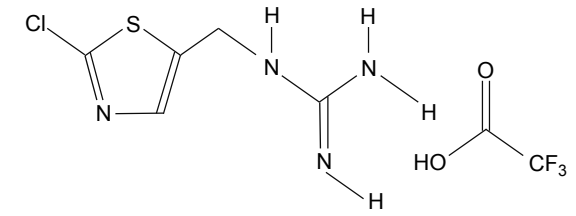

desmethyl-CLO

$^{1}$H NMR (400 MHz, $CD_3OD$)



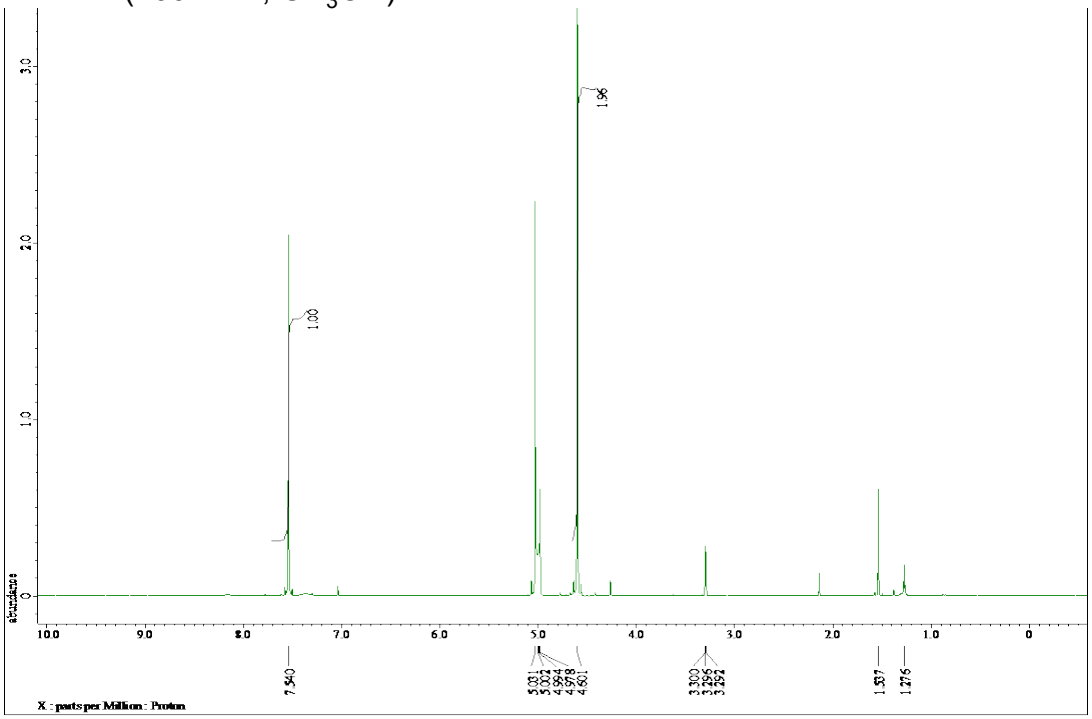

$^{13}$C NMR (100 MHz, $CD_3OD$)



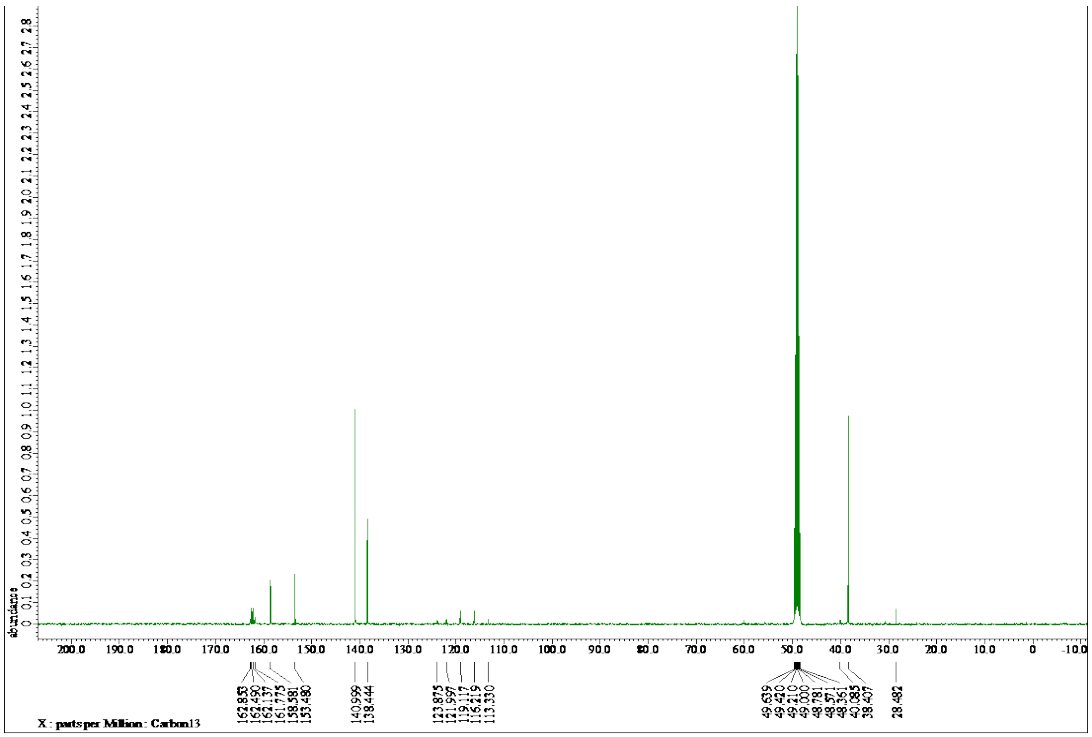


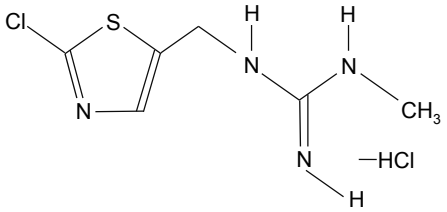

desmethyl-desnitro-CLO TFA salt

$^{1}$H NMR (400 MHz, $CD_3OD$)



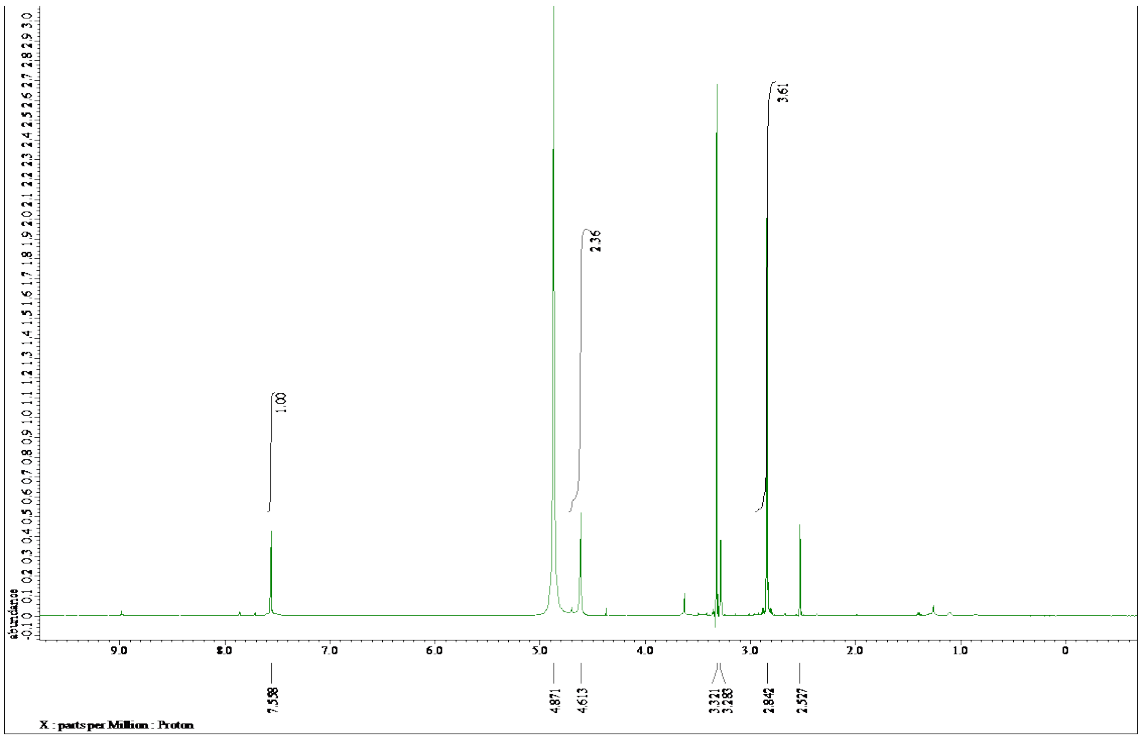

$^{13}$C NMR (100 MHz, $CD_3OD$)






desnitro-CLO HCl salt

$^{1}$H NMR (400 MHz, $CD_3OD$)

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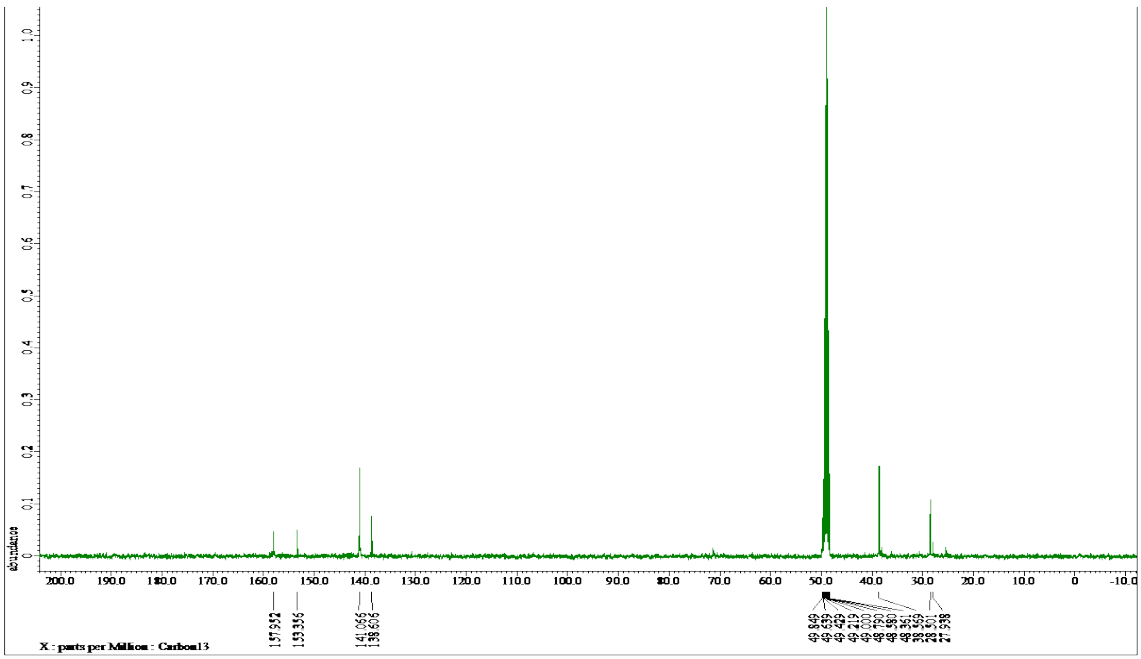
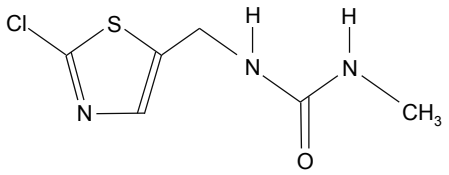
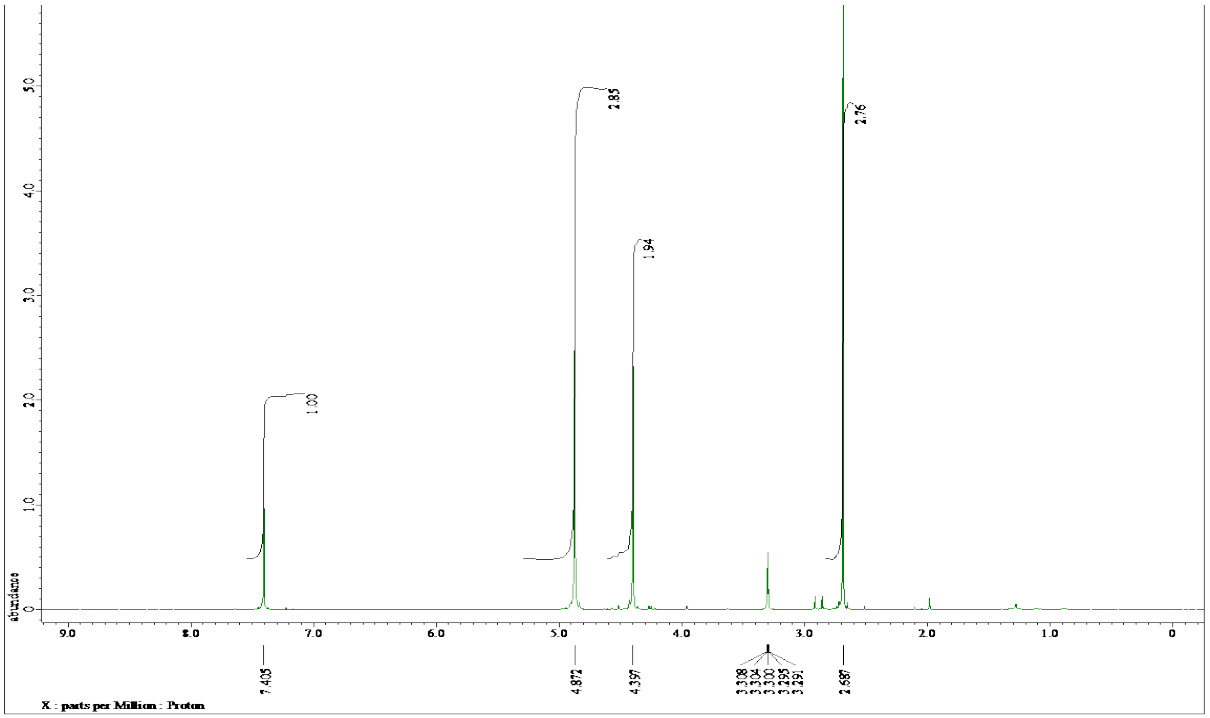





CLO-urea

$^{1}$H NMR (400 MHz, $CD_3OD$)




$^{13}$C NMR (100 MHz, $CD_3OD$)