

Characterization of the ileal muscarinic receptor system in 70-week-old Type II Goto-Kakizaki diabetic rats; effects of cyclohexenonic long-chain fatty alcohol

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Abstract

As gastrointestinal motility disorders are frequently reported in patients with diabetes, we attempted to clarify the effects of cyclohexenonic long-chain fatty alcohol in type 2 Goto-Kakizaki (GK) diabetic enteropathy. At 40 weeks of age male GK rats divided into three groups (treated with 0, 2 or 8 mg / kg of cyclohexenonic long-chain fatty alcohol; started at the age of 40 weeks). Age-matched male Wistar rats were used in this study. At 70 week of age the ileal functions were estimated by organ bath studies using 100 mM KCl and carbachol. The expression levels of muscarinic M₂ and M₃ receptor mRNAs in the ileum were investigated by real-time polymerase chain reaction (PCR). Treatment with cyclohexenonic long-chain fatty alcohol did not alter the diabetic status of the GK rats, i.e., body weight, serum glucose, and serum insulin levels, but significantly ameliorated diabetes-induced hypercontractility of the rat ileum caused by carbachol in a dose-dependent manner. Although there were no significant differences in the expression levels of muscarinic M₃ receptor mRNAs in any of the groups, cyclohexenonic long-chain fatty alcohol reversed the diabetes-induced up-regulation of intestinal muscarinic M₂ receptor mRNAs in treatment groups. These results indicate that cyclohexenonic long-chain fatty alcohol exerts its therapeutic effects on hypercontractility in the ileum of 70-week-old GK type 2 diabetic rats by ameliorating overexpression of muscarinic M₂ receptors.

Index Words: type 2 diabetes, muscarinic receptor, cyclohexenonic long-chain fatty alcohol, ileum, Goto-Kakizaki (GK) rat

1. Introduction

Gastrointestinal dysfunctions such as delayed gastric emptying, diarrhea, constipation, and abdominal pain occurs in many diabetic patients. These dysfunctions are primarily caused by diabetes-induced neuropathy (Hosking et al., 1978; Thomas et al., 1987), which upsets the balance between the cholinergic and adrenergic systems within the enteric nervous system, thereby disrupting the gastrointestinal tone and causing reduced peristalsis and dysmotility. The streptozotocin-induced diabetic rat, which is the most commonly used and well-investigated experimental model for type 1 diabetes, exhibits typical alterations of gastrointestinal function (Shinbori et al., 2006; Narimatsu et al., 2007). However, only limited information is available about enteropathy in rat models of type 2 diabetes.

The Goto-Kakizaki (GK) rat represents a spontaneous non-insulin-dependent diabetes model. GK rats are produced from normal Wistar rats by repetition of selective breeding, and are a widely accepted genetically determined rodent model for human type 2 diabetes. This genetic rat model is particularly relevant to human type 2 diabetes because it exhibits defects in glucose-stimulated insulin secretion, peripheral insulin resistance, and hyperinsulinemia as early as four weeks after birth, with impairment of insulin secretion and resulting hypoinsulinemia occurring at later stages (Goto et al., 1976; Goto and Kakizaki, 1981).

The tropical plant, *Hygrophilia erecta* Hochr., has been shown to contain some cyclohexenonic long-chain fatty alcohols that exert neurotrophic activities on cultured neurons from the cerebral cortex (Borg et al., 1987, 1990). Cyclohexenonic long-chain fatty alcohol has been found to directly increase the neurite extension as well as the biochemical differentiation of these neurons. In addition, we have reported that cyclohexenonic long-chain fatty alcohol prevented the progression of diabetes-induced alterations of the

trachea, aorta, and bladder (Sato et al., 2005; Shinbori et al., 2007; Saito et al., 2007). In other previous reports, we demonstrated that cyclohexenonic long-chain fatty alcohol ameliorated the progression of diabetes-induced dysfunction of the ileum in streptozotocin-induced diabetic rats (Shinbori et al., 2006; Narimatsu et al., 2007). In the previous reports, although cyclohexenonic long-chain fatty alcohol did not improve the general features or serum glucose and insulin levels of the streptozotocin-induced diabetic rats, it significantly improved the thickness of the ileal wall and hypercontractility of the rat ileum in response to carbachol in a dose-dependent manner, and the improvement of hypercontractility was accomplished without qualitative alteration of the muscarinic receptor system (Shinbori et al., 2006; Narimatsu et al., 2007). From these reports, we propose that cyclohexenonic long-chain fatty alcohol may prevent not only type 1 diabetic peripheral neuropathy in the ileum, but also type 2 diabetes; furthermore, these effects appear to take place independently of increases of insulin and serum glucose.

Therefore, the aims of this study were to investigate type 2 diabetes-induced changes in the muscarinic receptor system in the rat ileum, and to clarify the effects of cyclohexenonic long-chain fatty alcohol on possible alterations of the muscarinic receptor system in the type 2 rat diabetic ileum.

2. Materials and Methods

2.1. Animal models

All animal experiments were performed in accordance with the guidelines established by the Tottori University Committee for Animal Experimentation. Six-week-old male GK and Wistar rats were purchased from SLC (Shizuoka, Japan). All rats were kept under identical conditions, and had access to food and drinking water *ad libitum*. At the age of 12 weeks, seven Wistar and seven GK rats were used for preliminary functional studies. Then, the remaining rats were divided randomly into four groups (n = 6-8 each): a group of age-matched Wistar rats treated with vehicle (A), a group of diabetic GK rats treated with vehicle (B), a group of diabetic GK rats treated with cyclohexenonic long-chain fatty alcohol, 2,4,4-trimethyl-3-(15-hydroxypentadecyl)-2-cyclohexen-1-one, at a daily dose of 2 mg/kg intramuscularly (C), and a group of diabetic GK rats treated with cyclohexenonic long-chain fatty alcohol at a daily dose of 8 mg/kg intramuscularly (D). Figure 1 shows the chemical structure of this compound (Shinbori et al., 2007; 2008). A mixture of ethanol:saline:Tween 80 in the ratio 5:92.15:2.85 (the total volume was 1 ml/kg body weight) was used as a vehicle throughout this study (Saito et al., 2006; 2007, Shinbori et al., 2007; 2008). The drug or vehicle administrations were started at the age of 40 weeks in each animal. Upon reaching 70 weeks of the age, the rats were sacrificed with an overdose of pentobarbital (60 mg i.p.). Blood samples were collected from the vena cava, and the ileum was removed from each animal and placed in Krebs–Henseleit solution. These blood samples were frozen at -80°C until used. The rat ileum was immediately used for functional studies, and the rest of the tissue was frozen at -80°C for measurement of muscarinic M_2 and M_3 receptor mRNAs.

2.2. Serum glucose and insulin measurement

Serum glucose concentrations were measured by the hexokinase method using a Glucose C II kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's instructions in all groups. The insulin concentrations were also measured by a Rat Insulin ELISA kit (Merckodia AB, Uppsala, Sweden) according to the manufacturer's instructions.

2.3. Measurement of contractile force in the isolated ileum

The functional studies were performed according to our previous reports (Shinbori et al., 2006; Narimatsu et al., 2007). The ileum was rinsed with Krebs-Henseleit solution, the most distal 10 cm discarded, and the remaining ileum cut into approximately 1 cm segments. The ileal segments were mounted longitudinally between a hook in the bottom of the muscle chamber and a force displacement transducer in an organ bath (25 ml) containing Krebs-Henseleit solution, and bubbled with 5% CO₂ and 95% O₂ (37°C). One hook was suspended from a transducer (type 45196A; San-ei Instruments, Tokyo, Japan), and the lower hook was fixed to a plastic support leg to a micrometer (Mitutoyo, Tokyo, Japan). The ileum preparations were equilibrated unstretched for 30 min. A load of 1.0 g was applied to each strip by micrometer adjustment, and the load was readjusted to this level 30 min later. Changes in the tone of the strips were induced by means of a force transducer, and recorded on a personal computer (Macintosh G3; Apple Computer, Cupertino, CA) by use of Chart v 3.6.9 software and a PowerLab/16sp data acquisition system (AD Instruments, Castle Hill, Australia). Cumulative concentration-response curves to carbachol (10^{-8} – 3×10^{-5} M) and KCl (100 mM) were recorded.

2.4. Real-time polymerase chain reaction (PCR) of muscarinic M₂ and M₃ receptor mRNAs

Measurement of muscarinic receptor mRNAs was performed according to a method outlined in our previous reports (Shinbori et al., 2006; Narimatsu et al., 2007; Saito et al., 2007). Muscarinic M₂ and M₃ receptor mRNAs in the experimental ileum were measured by real-time PCR. The mRNAs were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The reverse transcriptase (RT) mixture (20 ml) containing 2 mg of total RNA was prepared and incubated at 37°C for 60 min according to a previously reported method (Shinbori et al., 2006; Narimatsu et al., 2007; Saito et al., 2007). Fifteen microliters of the mixture were used for real-time PCR, which was carried out using a Light Cycler thermal cycler system with a LightCycler-FastStart Hybridization Probe kit according to the manufacturer's instructions (Roche Diagnostics, Tokyo, Japan). The primers and probes were synthesized commercially by Nihon Gene Research Laboratories, Inc. (Sendai, Japan), and these sequences are shown in Table 1. The predicted product sizes of muscarinic M₂ and M₃ receptors were 148 bp and 149 bp, respectively. The primer and probe of the β -actin used were from the LightCycler-Primer/Probe Set (rat). β -actin was used as the internal standard. A total of 5 ml of solution was used for the sample.

2.5. Data analysis

The data for contractions induced by carbachol were normalized by those of 100 mM KCl. The EC₅₀ and E_{max} values were obtained by using a Macintosh computer (G3) loaded with Chart v3.6.9 software and a PowerLab/16sp data acquisition system. The expression of muscarinic M₂ and M₃ receptor mRNAs was quantified according to the expression of β -actin mRNAs in the experimental rat ileum. A statistical comparison of differences between groups was performed using analysis of variance and Fisher's multiple comparison tests.

Values of $P < 0.05$ were considered to indicate statistical significance.

2.6. Drugs and chemicals

Cyclohexenonic long-chain fatty alcohol,
2,4,4-trimethyl-3-(15-hydroxypentadecyl)-2-cyclohexen-1-one, was obtained from Meiji Milk
Products Co., Ltd. (Tokyo, Japan). All other chemicals used were of reagent grade.

3. Results

3.1. General features of the experimental rats

The general features of the experimental rats are shown in Figure 2 and Table 2. The GK diabetic rats showed significantly lower weight gain by the age of 10 weeks, as well as significantly lower weight gain during the experimental period compared with the controls ($p < 0.05$) (Figure 2). Significantly higher serum glucose levels were confirmed at the age of 12 weeks in the GK diabetic rats compared with the controls (153.4 ± 7.9 and 233.1 ± 12.3 mg/dl for the Wistar and GK rats, respectively; $p < 0.05$). The 70-week-old GK diabetic rats also showed significantly higher serum glucose and lower serum insulin levels than those of the control rats. Treatment with cyclohexenonic long-chain fatty alcohol did not improve the body weight gains during the observation periods, and had no effect on serum glucose or insulin levels.

3.2. Measurement of contractile force in the isolated ileum

The E_{max} and EC_{50} values for the contractile responses of the ileal smooth muscles to carbachol ($10^{-8} - 3 \times 10^{-5}$ M) and KCl (100 mM) were determined (Table 3). At the age of 12 weeks, there was no significant difference in E_{max} values between Wistar and GK rats. However, significant hypercontractility to carbachol was observed in the ileum of 70-week-old GK rats. Diabetes-induced ileal hypercontractility to carbachol was significantly ameliorated by the high-dose (8 mg/kg) treatment with cyclohexenonic long-chain fatty alcohol. The EC_{50} values obtained in response to carbachol indicated were not significantly difference among the four groups.

3.3. Real-time PCR analysis of muscarinic M_2 and M_3 receptor mRNAs

Figure 3 shows the expressions of muscarinic M₂ and M₃ receptor mRNAs in the ileum as assessed by real-time PCR. In the untreated diabetes group (group B), the expressions of muscarinic M₂ receptor mRNAs were significantly higher than those in the controls (group A). Treatment with both doses of cyclohexenonic long-chain fatty alcohol significantly improved the up-regulation of muscarinic M₂ receptor mRNAs. The expressions of muscarinic M₃ receptor mRNAs in the untreated diabetes group, tended to be higher than those in age-matched Wistar rats. However, because of the large variations in each group, these differences were not significantly different. Treatment with cyclohexenonic long-chain fatty alcohol tended to ameliorate diabetes-induced overexpression of muscarinic M₃ receptor mRNAs; the expression levels of muscarinic M₃ receptor mRNAs in GK rats treated with both doses were close to that of control Wistar rats. However, no significant differences in the expressions of muscarinic M₃ receptor mRNAs were found among the four groups.

4. Discussion

Several studies have examined contractile responses of ileal smooth muscle to acetylcholine or cholinergic agonists in streptozotocin-induced diabetes, and have alternatively reported that the responses are elevated (Shinbori et al., 2006; Narimatsu et al., 2007; Carrier and Aronstam, 1990; Anjaneyulu and Ramarao, 2002; Talubmook et al., 2003) or decreased (Lucas and Sardar, 1991; Coulson et al., 2002). Interestingly, Daneshgari and associates reported that diabetic bladders may undergo a transition from a compensated to a decompensated state and that this transition in the streptozotocin rat model may begin at 9 to 12 weeks after the induction of diabetes (Daneshgari et al., 2006). It may be that the same phenomenon occurs in the streptozotocin-induced diabetic rat ileum, and thus the conflicting results described above may have been due to the durations of the diabetic periods.

However, the majority of human diabetes is type 2. There are some reports indicating that neuropathy in type 1 diabetes is different from that in type 2 diabetes (Schmidt et al., 2003, 2004; Kamiya et al., 2005), and only limited information is available in regard to ileal dysfunction in type 2 diabetic models. In the present study, although there was no significant difference of E_{max} values for carbachol between Wistar and GK rats at the age of 12 weeks, we clearly demonstrated the hypercontractility of detrusor smooth muscle in response to carbachol in GK rats at the age of 70 weeks compared to age-matched Wistar rats. These data were similar to those for streptozotocin-induced diabetic rats previously reported (Shinbori et al., 2006; Narimatsu et al., 2007). These data are particularly interesting because early-stage streptozotocin-induced diabetic rats and late-stage GK rats show similar patterns of ileal dysfunction. In previous studies, we showed that significant alterations of the ileal muscarinic receptor system occurred over the eight-week period of streptozotocin-induced diabetes (Shinbori et al., 2006; Narimatsu et al., 2007). Thickening the ileal wall,

hypercontractility in response to carbachol, and up-regulations of the expressions of muscarinic M₂ and M₃ receptor mRNAs were observed in the streptozotocin-induced diabetic rat ileum. These alterations of the muscarinic receptor system in the rat ileum were quantitative rather than qualitative pharmacologically and biologically (Shinbori et al., 2006; Narimatsu et al., 2007).

As regards the role of muscarinic receptors, we have suggested that the rat ileum undergoes contractions mainly via the muscarinic M₃ receptor subtype using pharmacological methods (Narimatsu et al., 2007). However, based on the results of previous studies, we can not rule out the possibility that the muscarinic M₂ receptor subtype does not directly contribute to cholinergic contractions. In addition, Unno *et al.* reported results for muscarinic M₂-knockout, muscarinic M₃-knockout, muscarinic M₂/M₃-double knockout, and wild-type mice, demonstrating that muscarinic M₂ and M₃ receptors participate in mediating cholinergic contractions in the mouse ileum, with the latter subtype of receptors assuming a substantial role in this process (Unno et al., 2006). In order to clarify the mechanisms underlying type 2 diabetic enteropathy, we undertook to measure the expression levels of muscarinic M₂ and M₃ receptor mRNAs utilizing real-time PCR methods. In this study, we found that muscarinic M₂ receptor mRNAs were significantly up-regulated, and muscarinic M₃ receptor mRNAs were slightly up-regulated in the ileum of 70-week-old GK type 2 diabetic rats. The lack of statistical significance in the latter result may have been due to the small size of the groups we studied. The possible mechanisms of this up-regulation of muscarinic receptors are thought to include a decrease in cholinergic nerve density, or a defect in the neurotransmitter release mechanism (Miyamae et al., 2004; Tong et al., 1996). The diabetes-associated neuropathy may inhibit the release of acetylcholine from cholinergic nerves, in turn inducing the overexpression of muscarinic receptors in the diabetic ileal smooth muscle. Such

overexpression may enhance signaling downstream of these receptors and may increase smooth muscle contraction, according to the results of the present organ bath study.

Cyclohexenonic long-chain fatty alcohol was first reported to have neurotrophic activities on cultured neurons from the cerebral cortex (Borg et al., 1987, 1990). The C26-alcohol, N-hexacosanol, has been found to directly increase neurite extension as well as biochemical differentiation of these neurons. Watanabe and Miyagawa reported that cyclohexenonic long-chain fatty alcohol has beneficial effects on peripheral neuropathy and cystopathy in streptozotocin-induced diabetic rats (Watanabe and Miyagawa, 2002). They also demonstrated that diabetes-induced alteration of motor sciatic nerve conduction is normalized by treatment with cyclohexenonic long-chain fatty alcohol (Watanabe and Miyagawa, 2002). In more recent studies, our group has investigated mainly therapeutic and/or preventive effects on streptozotocin-induced cystopathy, enteropathy, angiopathy, nephropathy, and tracheal dysfunction (Narimatsu et al., 2007; Okada et al., 2008; Saito et al., 2006, 2007; Shinbori et al., 2006, 2007; Suzuki et al., 2006). We found that cyclohexenonic long-chain fatty alcohol has no effect on serum insulin or glucose levels in streptozotocin-induced diabetic animals, but that it has therapeutic and/or preventive effects on streptozotocin-induced cystopathy, enteropathy, angiopathy, nephropathy, and tracheal dysfunction. Recently, we have also reported that streptozotocin-induced diabetes caused alterations of amino acid components in the central nervous system of rats, and that treatment with cyclohexenonic long-chain fatty alcohol significantly ameliorated these alterations (Shinbori et al., 2008). These reports strongly suggest that cyclohexenonic long-chain fatty alcohol has therapeutic and/or preventive effects on not only peripheral neuropathy but also central neuropathy induced by streptozotocin. In this study, we also investigated the therapeutic effects of cyclohexenonic long-chain fatty alcohol on type 2 diabetes-induced ileal

alterations. We clearly demonstrated that cyclohexenonic long-chain fatty alcohol significantly ameliorated diabetes-induced hypercontractility of the rat ileum in response to carbachol, and that cyclohexenonic long-chain fatty alcohol reversed the diabetes-induced up-regulation of intestinal muscarinic M₂ receptor mRNAs in the rats. From this study, although the precise mechanisms are unclear, it is clear that cyclohexenonic long-chain fatty alcohol reduces hypercontractility of the ileum of 70-week-old GK type 2 diabetic rats by ameliorating overexpression of muscarinic M₂ receptor proteins and mRNAs. The physiological and pharmacological role of cyclohexenonic long-chain fatty alcohol in type 2 diabetes mellitus, however, remains unclear and warrants further study.

In conclusion, our data indicate that cyclohexenonic long-chain fatty alcohol ameliorates hypercontractility in the ileum of 70-week-old GK type 2 diabetic rats by ameliorating overexpression of muscarinic M₂ receptor and possibly M₃ receptor subtypes.

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Figure legends

Figure 1. The chemical structure of cyclohexenonic long-chain fatty alcohol, 2,4,4-trimethyl-3-(15-hydroxypentadecyl)-2-cyclohexen-1-one.

Figure 2. Body weight in GK and Wistar rats.

A: Wistar rats; B: GK rats treated with vehicle; C: GK rats treated with 2 mg/kg of cyclohexenonic long-chain fatty alcohol; D: GK rats treated with 8 mg/kg of cyclohexenonic long-chain fatty alcohol. Data are shown as the means \pm SEM of six to eight separate determinations in each group. *Significantly different from the other groups of age-matched rats.

Figure 3A and 3B. Expressions of muscarinic M₂ and M₃ receptor mRNAs, respectively.

A: Wistar rats; B: GK rats treated with vehicle; C: GK rats treated with 2 mg/kg of cyclohexenonic long-chain fatty alcohol; D: GK rats treated with 8 mg/kg of cyclohexenonic long-chain fatty alcohol. All data were normalized by b-actin. Data are shown as the means \pm SEM of six to eight separate determinations in each group. *Significantly different from the other groups of age-matched rats.

Table 1

Oligonucleotides of primers and hybridization probes for amplification of muscarinic M₂ and M₃ receptors.

Muscarinic M₂ Receptor Subtype	
primer	Forward 5' CCACTCCAGATGACAACT 3' Reverse 5' GGCTACAACGTTCTGCTTT 3'
Probe	5' -CCAACTAGTTCTACAGTGGTACTCGTTGGGGTG-3' -Fluorescein 3' Label: Fluorescein 5' -LCRed640-ACACATCACCTTTTGGGCCCTTGGTGAC-3' -phosphorylation 5' Label: LCRed640 3' Label: phosphorylated
Muscarinic M₃ Receptor Subtype	
primer	Forward 5' GGACTGTGGATGTGGAGAG 3' Reverse 5' CGAGGAGTTGGTGCAGA 3'
Probe	5' -CCAGAAAGACATGGGTGATGGTGACAAC-3' -Fluorescein 3' Label: Fluorescein 5' -LCRed640-GTCAGAAAGGATTTGACCAAGCTTCCCATCC-3' -phosphorylation 5' Label: LCRed640 3' Label: phosphorylated

Table 2

General features of the experimental rats

	Body Weight (g)		Serum glucose (mg/dl)	Serum insulin ($\mu\text{g/L}$)
	40 weeks old	70 weeks old		
A	525.7 \pm 20.7	551.4 \pm 22.3	132.2 \pm 5.9	2.51 \pm 0.33
B	419.3 \pm 4.0 ^a	394.3 \pm 3.7 ^a	208.1 \pm 8.5 ^a	0.53 \pm 0.10 ^a
C	416.4 \pm 8.3 ^a	400.0 \pm 8.7 ^a	217.3 \pm 16.4 ^a	0.54 \pm 0.10 ^a
D	409.3 \pm 5.6 ^a	387.1 \pm 8.1 ^a	209.1 \pm 6.1 ^a	0.64 \pm 0.15 ^a

A: control rats, B: diabetic rats, C: diabetic rats treated with 2 mg/kg cyclohexenonic long-chain fatty alcohol. D: diabetic rats treated with 8 mg/kg cyclohexenonic long-chain fatty alcohol. Data are shown as mean \pm S.E.M. of 6 to 8 separated determinations in each group. ^a Significantly different from group A. (P<0.05)

Table 3
Functional studies in the experimental rats

	E_{\max}/KCl	$\text{EC}_{50} (10^{-7} \text{ M})$
12-week-old		
Wistar	1.36 ± 0.09	4.25 ± 0.81
GK	1.28 ± 0.06	4.37 ± 0.74
70-week-old		
A	1.28 ± 0.07	4.32 ± 0.87
B	1.60 ± 0.13^a	6.33 ± 1.46
C	1.52 ± 0.07^a	4.13 ± 0.43
D	1.36 ± 0.05	4.65 ± 1.18

The E_{\max} values to the contractile response to carbachol and KCl (100mM) were determined. The EC_{50} values with respect to carbachol. A: control rats, B: diabetic rats, C: diabetic rats treated with 2 mg/kg cyclohexenonic long-chain fatty alcohol. D: diabetic rats treated with 8 mg/kg cyclohexenonic long-chain fatty alcohol. Data are shown as mean \pm S.E.M. of 6 to 8 separated determinations in each group. ^a

Significantly different from group A. ($P < 0.05$)

Figure 1

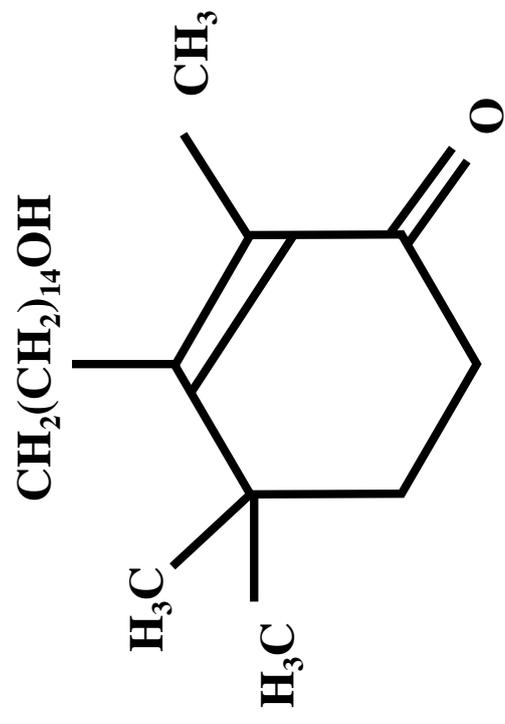


Figure 2

