

**Table I.** Effect of Xanthine and Xanthine Oxidase on DNA Cleavage Measured by Inhibition of *E. Coli* Polymerase I

incubation conditions <sup>a</sup>	% activity <sup>b</sup>
control	100
OP (12.5 $\mu$ M), Cu <sup>2+</sup> (1 $\mu$ M)	103
xanthine (10 <sup>-4</sup> M), xanthine oxidase (6.7 $\times$ 10 <sup>-3</sup> units/mL), Cu <sup>2+</sup> (1 $\mu$ M)	80
xanthine (10 <sup>-4</sup> M), xanthine oxidase (6.7 $\times$ 10 <sup>-3</sup> units/mL), Cu <sup>2+</sup> (1 $\mu$ M), catalase (50 $\mu$ g/mL)	97
xanthine (10 <sup>-4</sup> M), xanthine oxidase (6.7 $\times$ 10 <sup>-3</sup> units/mL), OP (12.5 $\mu$ M), Cu <sup>2+</sup> (1 $\mu$ M)	4
xanthine (10 <sup>-4</sup> M), xanthine oxidase (6.7 $\times$ 10 <sup>-3</sup> units/mL), OP (12.5 $\mu$ M), Cu <sup>2+</sup> (1 $\mu$ M), catalase (50 $\mu$ g/mL)	84

<sup>a</sup> All incubation mixtures contained [<sup>3</sup>H]TTP (50  $\mu$ M), dATP (50  $\mu$ M), and poly(dA-T) (10  $\mu$ g/mL). Incubation time, 3 min.

<sup>b</sup> Percent activity, 100 s after addition of *E. coli* polymerase I (see ref 1 for details).

methane, another effective superoxide trap, was used to demonstrate the presence of superoxide in the stoichiometric oxidation of the crystalline perchlorate salt of (OP)<sub>2</sub>Cu<sup>+</sup>.<sup>15</sup> Tetranitromethane could not be used in the in situ reduction and reoxidation of (OP)<sub>2</sub>Cu<sup>+</sup> because it reacts with free sulfhydryl groups. (OP)<sub>2</sub>Cu<sup>+</sup>, prepared by the addition of OP to an acetonitrile solution of Cu(CH<sub>3</sub>CN)<sub>4</sub>ClO<sub>4</sub>,<sup>16</sup> is indistinguishable in its reactivity to the crystalline cuprous complex described above. If thiol or ascorbic acid is added to the aerobic aqueous solution used to oxidize either of these complexes, the steady-state reduction of NBT is observed.

The cupric complexes of 2,2',2''-terpyridine and bipyridine produce diffusible superoxide at rates comparable to (OP)<sub>2</sub>Cu<sup>2+</sup> in the presence of thiol and O<sub>2</sub>. Since the 1,10-phenanthroline-copper complex is unique in causing inhibition of DNA polymerase by cleaving DNA,<sup>17,18</sup> diffusible superoxide cannot be directly responsible for the cleavage reaction. This result is supported by the failure of superoxide generated by xanthine and xanthine oxidase to cleave DNA and inhibit the enzyme (Table I). However, xanthine and xanthine oxidase can potentiate the cleavage of DNA by (OP)<sub>2</sub>Cu<sup>2+</sup> in a reaction that is blocked by catalase (Table I). Since the DNA cleavage reaction and its associated *E. coli* polymerase I inhibition are blocked by 2,9-dimethyl-1,10-phenanthroline, a cuprous ion specific chelator,<sup>17,18</sup> the potentiation by superoxide can be readily explained if (OP)<sub>2</sub>Cu<sup>+</sup> and H<sub>2</sub>O<sub>2</sub> are the essential reactants for the DNA cleavage reaction. Superoxide can enhance the concentration of (OP)<sub>2</sub>Cu<sup>+</sup> by reduction of (OP)<sub>2</sub>Cu<sup>2+</sup> (eq 3.1 and 3.2)<sup>9</sup> and generate hydrogen peroxide by its spontaneous dismutation (eq 3.3). Like any other reducing agent, superoxide enhances the DNA cleavage reaction by increasing the concentrations of (OP)<sub>2</sub>Cu<sup>+</sup> and hydrogen peroxide.

In this report, we have established for the first time that the oxidation of the cuprous complex of 1,10-phenanthroline and related ligands by oxygen proceeds via a superoxide intermediate. However, it is clear that diffusible superoxide is not directly involved in the DNA cleavage reaction caused by (OP)<sub>2</sub>Cu<sup>2+</sup> and thiol under aerobic conditions. The ability of superoxide generators such as xanthine and xanthine oxidase to substitute for thiol in the cleavage reaction can be fully explained in terms of (OP)<sub>2</sub>Cu<sup>+</sup> and hydrogen peroxide being essential reactants for the breakdown of the DNA. Studies in progress have confirmed this conclusion by showing that addition of hydrogen peroxide to an anaerobic solution of (OP)<sub>2</sub>Cu<sup>+</sup> causes the cleavage of DNA within 1 min.

The exclusive reactivity of the 1,10-phenanthroline complex is striking. It may relate to its ability to intercalate into DNA in a unique orientation. Once bound to the polynucleotide, it may react directly with hydrogen peroxide to yield hydroxyl radicals via chemistry analogous to that of Fenton's reagent,<sup>19</sup> or it may facilitate the nucleophilic attack of hydrogen peroxide on the phosphodiester bond.

In passing, it is interesting to note that (OP)<sub>2</sub>Cu<sup>2+</sup> is widely used to cross-link proteins via disulfide linkages.<sup>20</sup> Superoxide must be produced during this reaction and may lead to potential complexities in interpretation, especially when it is formed in a hydrophobic milieu where it is a potent nucleophile.<sup>21</sup>

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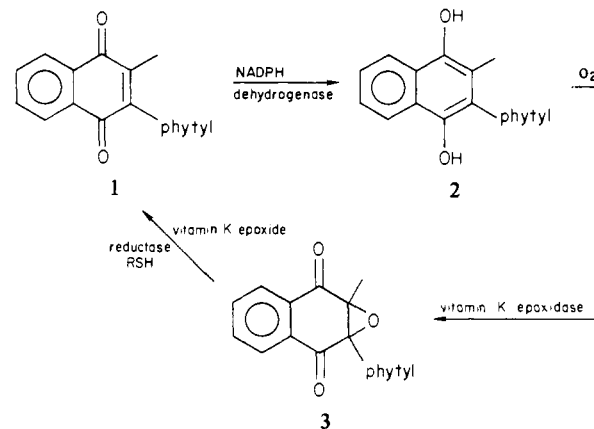
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### A Model for a Molecular Mechanism of Anticoagulant Activity of 3-Substituted 4-Hydroxycoumarins

Sir:

Vitamin K (1), a fat-soluble vitamin essential for the coagulation of blood, has been shown to be required for the biosynthesis of active prothrombin and other plasma clotting factors.<sup>1</sup> It has



been established that the active form of the vitamin is the reduced (hydroquinone) form<sup>2</sup> (2) and that an O<sub>2</sub>-dependent epoxidase catalyzes the conversion of 2 to vitamin K 2,3-epoxide (3).<sup>3</sup> The epoxidase activity has been linked with the activation of prothrombin and the coagulation of blood.<sup>4</sup> It has been suggested that vitamin K epoxide is biosynthesized as a means of intracellular storage of an inactive form of the vitamin.<sup>5</sup> Because of the

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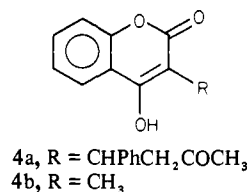
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relatively slow uptake of vitamin K into the cell, a ready supply of vitamin K precursor would be available to the cell for immediate utilization. Vitamin K epoxide reductase activity has been observed and is responsible for the conversion of the epoxide back to vitamin K.<sup>6</sup> The reported data suggest that 3-substituted 4-hydroxycoumarins [e.g., warfarin (**4a**)] inhibit this reductase;<sup>7</sup>

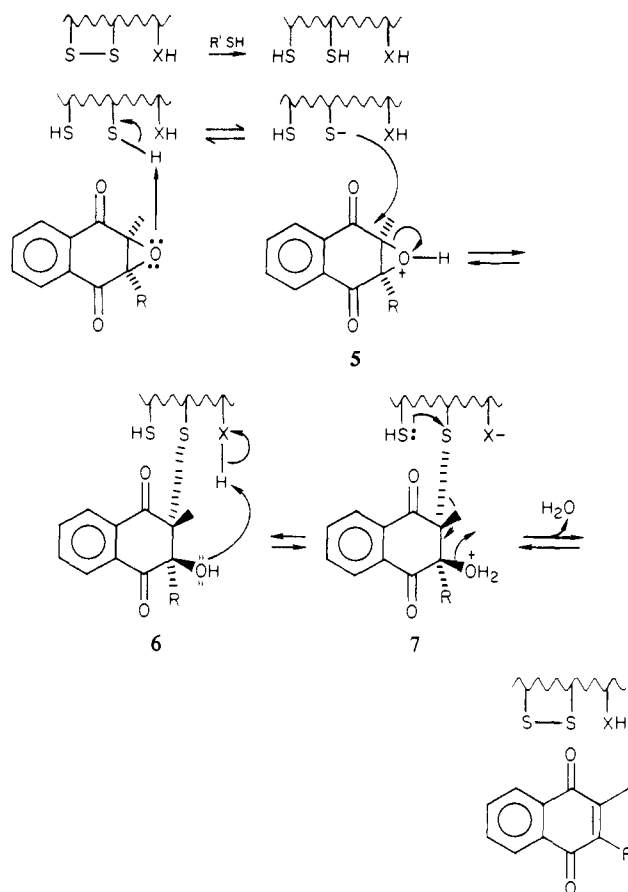


recently, the reductase was isolated free of epoxidase activity, and **4a** was shown to be a potent inhibitor.<sup>7c</sup> This suggests that the anticoagulant activity exhibited by these coumarin compounds results from the blockage of the regeneration of vitamin K from the epoxide within the cell. If this enzyme is needed only to regenerate vitamin K from the epoxide, then feeding warfarin-treated animals with vitamin K should produce a coagulation response. This is, in fact, the case.<sup>8</sup> The molecular mechanism of anticoagulant action of **4** is not known; we report here a chemical model for a proposed mechanism of these compounds.

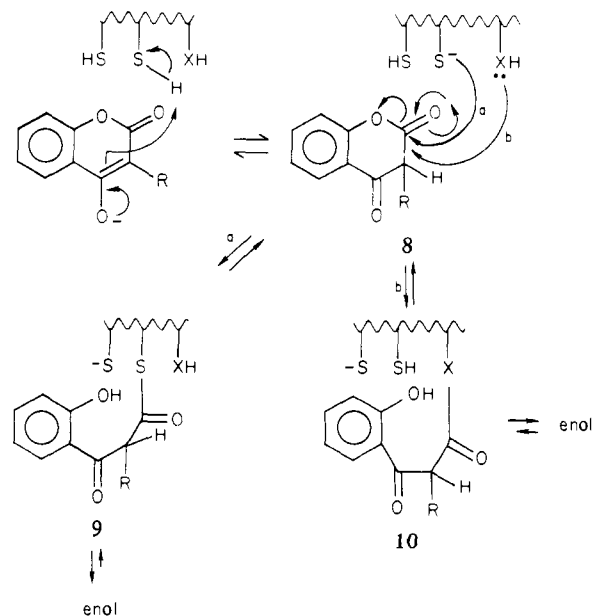
We propose that these anticoagulants are mechanism-based inactivators of vitamin K epoxide reductase. A mechanism-based (or suicide) inactivator<sup>9</sup> is an unreactive molecule which bears a structural similarity to the substrate or product of the target enzyme. It is converted by the enzyme into a reactive molecule which then forms a covalent bond with an essential moiety of the enzyme. The mechanism for the reduction of vitamin K epoxide to vitamin K is not known. However, the enzyme requires added thiol for activity, and this requirement could not be substituted by NADH.<sup>7c</sup> A plausible mechanism can be drawn to account for these observations and is shown in Scheme I. The oxidized enzyme is converted into the active reduced form by added thiol. Enzyme-catalyzed protonation of the epoxide generates a reactive epoxide (**5**) which can undergo backside attack by a sulfhydryl group, leading to enzyme-bound substrate (**6**). Protonation of the alcohol generates an active leaving group for reductive elimination which would result from sulfhydryl attack on the substrate-bound sulfur (**7**), thus yielding vitamin K and regenerating the oxidized enzyme. The principal steps in this mechanism have literature chemical precedence. Epoxides are known to form  $\beta$ -hydroxy sulfides with thiols.<sup>10</sup> Reductive elimination was proposed as the mechanism for the conversion of  $\beta$ -keto sulfides to ketones by thiols.<sup>11</sup>

The structure of 3-substituted 4-hydroxycoumarins is quite similar to that of vitamin K, the product yielded by vitamin K epoxide reductase. Thus, it is reasonable that these compounds would bind to this enzyme. Enzyme-catalyzed protonation of the double bond of these compounds, analogous to the corresponding protonation of the epoxide of substrate, leads to 3-substituted 2,4-chromandiones (**8**), the unstable tautomeric form of **4** (Scheme II). These unknown compounds, substituted *o*-ketophenyl lactones (**8**), would be expected to be susceptible to nucleophilic attack at the lactone carbonyl and to have reactivities intermediate between those of phenyl esters and *o*-nitrophenyl esters. These

**Scheme I.** Proposed Mechanism of Action of Vitamin K Epoxide Reductase (R = Phytyl)



**Scheme II.** Proposed Mechanism of Inactivation of Vitamin K Epoxide Reductase by 3-Substituted 4-Hydroxycoumarins



reactive compounds, then, could covalently modify the enzyme to give **9** or **10** (Scheme II) by acylation of an active-site thiol or other active-site nucleophile.<sup>12</sup>

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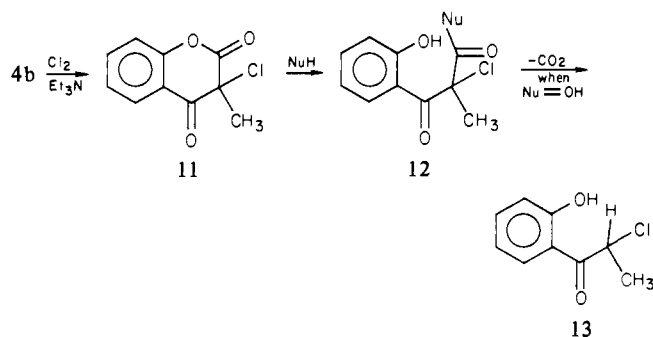
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(12) It has been reported<sup>13</sup> that with a single dose of warfarin in humans maximum anticoagulant activity levels were attained after 36–72 h. Blood coagulability then rose slowly over several days to the normal level. This recovery could be a result of regeneration of enzyme activity as a result of new protein biosynthesis or as a result of slow hydrolysis of the acyl covalent bond formed during the proposed inactivation.

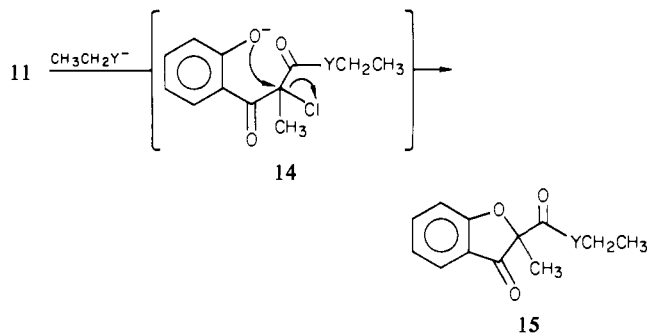
The appropriate model system for this proposed mechanism must be consistent with the following criteria: (1) 3-substituted 4-hydroxycoumarins (**4**) must be unreactive toward nucleophiles, (2) they must be converted into reactive molecules by an electrophilic substitution at the 3 position, and (3) the 3,3-disubstituted 2,4-chromandiones generated in the electrophilic reaction must react readily with nucleophiles at the lactone carbonyl. Numerous 3-substituted 4-hydroxycoumarins have been prepared and tested for their anticoagulant activity.<sup>14</sup> The potency of the compounds depends upon the structure of the 3-substituent. We believe that this substituent is important to the stability of the E-I complex, but it is not involved in the chemistry of the inhibition. Therefore, we have synthesized 3-methyl-4-hydroxycoumarin (**4b**)<sup>15</sup> as our model compound for this class of drugs. Treatment of **4b** with excess hot aqueous sodium hydroxide, sodium ethoxide in refluxing ethanol, *n*-propylamine in refluxing THF, or refluxing ethanol overnight, followed by reacidification, yielded >90% recovery of **4b**. This compound, then, is not reactive toward nucleophiles, probably because the most facile reaction is removal of the 4-hydroxyl proton to give a highly resonance-stabilized anion.

Since the tautomer of **4b** is not stable, we carried out a different electrophilic substitution reaction on **4b** to mimic the protonation step and to prepare a stable 2,4-chromandione for use as a model for **8**. Electrophilic substitution of **4b** by excess dry chlorine in dry THF containing 1 equiv of triethylamine at 0 °C produced an immediate white precipitate (triethylamine·HCl); anhydrous workup afforded 3-chloro-3-methyl-2,4-chromandione (**11**)<sup>16</sup> in 86% yield. Compound **11** was used as a model for the proposed reactive acylating agent (**8**).



Treatment of **11** in dry dioxane with 1 equiv of *n*-propylamine (a model for the reaction of **8** with an amino-containing residue on the enzyme) gave an immediate reaction, affording the corresponding amide, *o*-[2-(*N*-propylcarboxamido)-2-chloropropionyl]phenol (**12**, Nu = NHPr),<sup>17</sup> in quantitative yield. Compound **11** was solvolyzed in refluxing ethanol (a model for the reaction of **8** with a hydroxyl-containing residue on the enzyme) to *o*-(2-carboxy-2-chloropropionyl)phenol (**12**, Nu = OEt)<sup>18</sup> in 97% yield. Attempted purification of this compound by vacuum distillation led to isolation of a mixture of **12** (Nu =

OEt) plus **11**. When compound **12** (Nu = OEt) was heated at 120–130 °C for 33 h, compound **11** was obtained. This solvolysis reaction apparently is reversible.<sup>19</sup> Treatment of **11** in 2:1 dry dioxane/ethanol with 1 equiv of sodium ethoxide gave an immediate reaction producing not **12** (Nu = OEt) but instead compound **15**<sup>20</sup> (Y = O) in 88% yield. This compound could arise



by the expected reaction with ethoxide followed by an intramolecular S<sub>N</sub>2-type displacement of chloride (**14**). A similar reaction was reported recently.<sup>21</sup> The analogous reaction of **11** with ethanethiol (a model for the reaction of **8** with a cysteine residue) and triethylamine gave the thioester **15**<sup>22</sup> (Y = S) in 82% yield. A 250 mM solution of **11** in 50% aqueous dioxane underwent hydrolysis and decarboxylation<sup>23</sup> at 35 °C in several hours to give *o*-(2-chloropropionyl)phenol (**13**).<sup>24</sup> Hydrolysis of a 50 mM solution of **11** in 0.6 M Hepes buffer in 1:1 dioxane/D<sub>2</sub>O, pD 7.4, and 35 °C occurred with a half-life of about 3 min. The above reactions indicate that 3,3-disubstituted 2,4-chromandiones are, in fact, highly reactive compounds at the lactone carbonyl group.

These experiments provide chemical support for a mechanistic hypothesis that the anticoagulant activity of 3-substituted 4-hydroxycoumarin drugs is a result of mechanism-based inactivation of vitamin K epoxide reductase.

**Acknowledgments.** I thank Professor Paul A. Bartlett (Berkeley) for suggesting the synthesis of compound **11**, Richard E. Radak for preparing the 3-methyl-4-hydroxycoumarin used in this work, and Biron O. Lim and W. Gregory Ward for carrying out some exploratory reactions.

(19) Ethanolysis of 3-allyl-3-methyl-2,4-chromandione led to an equilibrium mixture of starting material: *o*-(2-carboxy-2-allylpropionyl)phenol (~50:50).

(20) Compound **15** (Y = O) had the following properties: NMR (CDCl<sub>3</sub>) δ 1.21 (t, 3 H), 1.71 (s, 3 H), 4.20 (q, 2 H), 6.95–7.25 (m, 2 H), 7.45–7.8 (m, 2 H); IR (film) 2990 (m), 1750 (s), 1720 (s), 1605 (s), 1470 (m), 1460 (m), 1260 (s), 1125 (m), 1090 (m) cm<sup>-1</sup>; colorless oil, bp 84 °C (0.15 mmHg). Anal. Calcd for C<sub>12</sub>H<sub>12</sub>O<sub>4</sub>: C, 65.45; H, 5.49. Found: C, 65.46; H, 5.56.

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(22) Compound **15** (Y = S) had the following properties: NMR (CDCl<sub>3</sub>) δ 1.13 (t, 3 H), 1.79 (s, 3 H), 2.60 (q, 2 H), 6.95–8.0 (m, 4 H); IR (KBr) 1770 (s), 1760 (s), 1690 (s), 1685 (sh), 1608 (s), 1458 (s) cm<sup>-1</sup>; white crystals, mp 49–50 °C. Anal. Calcd for C<sub>12</sub>H<sub>12</sub>O<sub>3</sub>S: C, 61.00; H, 5.12; S, 13.57. Found: C, 60.86; H, 5.07; S, 13.64.

(23) When the reaction is followed by NMR, a singlet 0.1 ppm upfield of the methyl proton singlet of starting material begins to appear. While that peak gets larger, the doublet of the hydrolysis and decarboxylation product, *o*-(2-chloropropionyl)phenol (**13**), begins to appear. Ultimately, the upfield singlet disappears, and only the doublet remains. Although the compound responsible for the upfield singlet has not been stable enough to isolate, it is presumed to be the initial hydrolysis product, *o*-(2-carboxy-2-chloropropionyl)phenol.

(24) Compound **13** has the following properties: NMR (CDCl<sub>3</sub>) δ 1.72 (d, 3 H), 5.27 (q, 1 H), 6.7–7.9 (m, 4 H), 11.8 (s, 1 H); IR (film) 3100 (b), 1640 (s), 1610 (m), 1575 (m), 1480 (m), 1445 (m) cm<sup>-1</sup>; pale yellow oil, bp 74 °C (0.4 mmHg) [lit.<sup>25</sup> 126–128 °C (10 mmHg)].

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(16) Compound **11** had the following properties: NMR (CDCl<sub>3</sub>) δ 1.99 (s, 3 H), 7.1–8.1 (m, 4 H); IR (KBr) 1780 (s), 1705 (s), 1607 (s), 1455 (s), 1320 (s), 1215 (s), 1090 (s) cm<sup>-1</sup>; shiny white needles, mp 140–140.5 °C. Anal. Calcd for C<sub>10</sub>H<sub>7</sub>ClO<sub>3</sub>: C, 57.03; H, 3.35; Cl, 16.83. Found: C, 56.84; H, 3.47; Cl, 16.78.

(17) Compound **12** (Nu = NHPr) had the following properties: NMR (CDCl<sub>3</sub>) δ 0.95 (t, 3 H), 1.52 (m, 2 H), 1.96 (s, 3 H), 3.28 (m, 2 H), 6.6–7.8 (m, 5 H), 11.6 (s, 1 H); IR (KBr) 3320 (s), 3200 (b), 1640 (s), 1610 (m), 1525 (m), 1435 (m) cm<sup>-1</sup>; shiny white needles, mp 88.5–89 °C. Anal. Calcd for C<sub>13</sub>H<sub>16</sub>ClNO<sub>3</sub>: C, 57.89; H, 5.98; N, 5.19; Cl, 13.14. Found: C, 57.84; H, 5.93; N, 5.04; Cl, 12.88.

(18) Compound **12** (Nu = OEt) before distillation had the following properties: NMR (CDCl<sub>3</sub>) δ 1.11 (t, 3 H), 1.98 (s, 3 H), 4.22 (q, 2 H), 6.6–7.85 (m, 4 H), 11.55 (s, 1 H); IR (film) 3100 (b), 2990 (s), 1750 (s), 1720 (s), 1640 (s), 1610 (m), 1575 (m), 1480 (m), 1445 (s) cm<sup>-1</sup>; pale yellow oil.