Enzyme Catalysed Preparation of 6-*O*-Acylglucopyranosides

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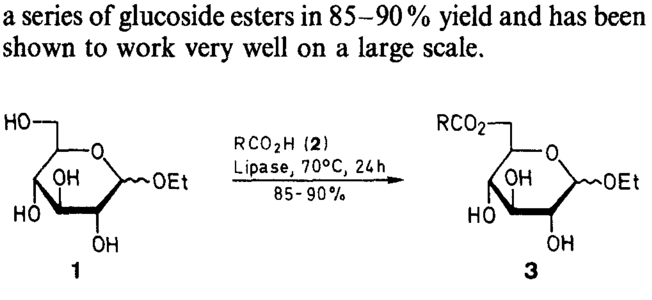
6-*O*-Monoesters of alkyl glucopyranosides have been prepared on a large scale in a more than 90% yield by direct enzyme-catalysed esterification of glucopyranosides with long chain fatty acids in a solvent-free process.

Fatty acid esters of carbohydrates have potentially important applications in detergents, food and feed, cosmetics and pharmaceuticals because of their surface-active properties. So far, however, attempts to prepare these esters from inexpensive, renewable and easily accessible starting materials have not been successful:[1] direct coupling of fatty acids and sugars has not given rise to products in appreciable yields and purity, while syntheses involving protection and deprotection procedures have been demonstrated not to be economically feasible with respect to the uses of the esters envisioned.[2] More recently, enzyme-catalysed transformations have been used to affect specific modifications of carbohydrates.[3] For example, monoacylated sugars have been prepared by lipase-catalysed transesterification using either activated esters in pyridine[4] or protease-catalysed esterification in DMF.[5] Direct enzymatic esterification of sucrose or glucose with a fatty acid in an aqueous medium has likewise been tried but the yield and selectivity obtained were low.[6]

On this basis we have developed a high-yielding enzyme-catalysed synthesis of 6-*O*-monoesters of simple alkyl glucopyranosides, (Scheme A).[7] This reaction was carried out simply by mixing the starting glucoside **1** with a fatty acid at 70 °C in the presence of an immobilised lipase. The water generated in the reaction was removed *in vacuo*. This convenient solvent-free process was used to prepare a series of glucoside esters in 85–90% yield and has been shown to work very well on a large scale.



2,3	a	b	c	d	e	f
R	n-C_7H_{15}	n-C_9H_{17}	n-C_4H_{19}	n-$C_{13}H_{21}$	n-$C_{15}H_{23}$	n-$C_{17}H_{25}$

Scheme A

The reactivity of glucosides in lipase-catalysed esterifications has been found to vary with the nature of the aglycon group. Thus, we recently found ethyl glucopyranoside to react more slowly than the propyl and butyl glucopyranoside but considerably faster than either methyl glucopyranoside or glucose.[7] Presumably, the difference observed is due to an improved solubility of the glucosides carrying longer alkyl chains as aglycons. To further investigate the substrate specificity of the enzymes and also the properties of the products we tested anomerically pure ethyl glucopyranosides as substrates in small-scale reactions (Tables 1 and 2). Pure ethyl β-D-glucopyranoside was synthesized from tetra-*O*-acetyl-α-D-glucopyranosyl bromide and ethanol by Koenig–Knorr glycosylation or by direct enzymatic glucosylation of glucose using a β-glucosidase as catalyst. Pure ethyl α-D-glucopyranoside was prepared from a mixture of α- and β-anomers by selective hydrolysis of the β- form with β-glucosidase. In the preparation of the pure α- and β-anomers of ethyl glucopyranoside esters Lipozyme®, a lipase from *Mucor miehei* was used as the catalyst. Ethyl β-D-glucopyranoside exhibited about twice the reactivity of the ethyl α-D-glucopyranoside. This may be explained by the different polarities of the anomers and thereby their solubilities in fatty acids or as a substrate selectivity of the enzyme in preference of the β-anomer. A similar preference was observed when using the immobilized lipase preparation from *Candida antarctica*[8] as catalyst. In our further experiments this enzyme was preferentially used, especially in large-scale preparations, because of its better performance.

The lipases from *Candida antarctica* are very heat-stable and durable enzymes. Two lipase components (A und B) have been isolated;[8] the B enzyme being efficient in synthesis and responsible for the highly regioselective esterification reaction. The selectivity in favour of formation of the 6-*O*-monoester with the purified B-component

Table 1. Alkyl Esters of Ethyl Glucopyranosides **3** Prepared[a]

Product	Yield (%)[b,c]		mp (°C)	$[\alpha]_D^{25}$ (c, $CHCl_3$)	CMC[d] ($\times 10^{-5}$ mol L^{-1})	γ-min[e] (mN/m)
	mono	di				
α-**3a**	44	12	syrup	70.9 (1.1)	> 500	< 30
α-**3b**	68	11	syrup	63.0 (1.3)	69	31.2
α-**3c**	64	7	syrup	60.7 (1.4)	4.7	31.7
α-**3d**	70	7	42–45	54.5 (1.3)	2.9	37.5
α-**3f**	70	10	45–51	49.9 (1.1)	3.9	46.5
α-**3g**	79	6	54–56	48.4 (1.1)		
β-**3a**	70	6	79–80	−52.4 (1.0)	> 500	< 30
β-**3b**	75	4	82–83	−49.7 (1.0)	110	30.6
β-**3c**	79	0.3	76–78	−46.7 (1.1)	6.9	32.4
β-**3d**	77	4	82–83	−43.5 (1.2)	24	32.7
β-**3e**	76	11	90–91	−42.4 (1.0)	4.0	43.4
β-**3f**	78	15	93–94	−39.7 (1.0)	2.6	43.3

[a] In the preparation of this series of anomerically pure compounds we used Lipozyme® as catalyst. This enzyme is not as selective and efficient a catalyst as a lipase from *Candida antarctica* which we used throughout our further investigations of the reaction.

[b] The reactions were stopped at a conversion of about 75–85% and the yields were determined by HPLC (mol %). The desired conversion was reached in 24–72 h, except for the short chain (C_8–C_{10}) acids which required 8–10 days.

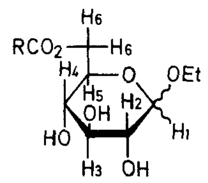
[c] Mono denotes ethyl 6-*O*-monoacyl-D-glucopyranoside **3a–f** and di denotes ethyl 2,6-*O*-diacyl-D-glucopyranoside **3a–c**.

[d] Critical micelle concentration.

[e] Minimal surface tension.

[f] The product was insoluble.

Table 2. Representative ^{1}H- and ^{13}C-NMR Spectroscopic Data for Alkyl Glucopyranosides α-**3a**, α-**3c**



Product	^{1}H-NMR ($CDCl_3$/TMS) δ, J(Hz)	^{13}C-NMR ($CDCl_3$) δ
α-**3a**	0.89 (t, 3H), 1.28 (m, 19H), 1.62 (m, 2H), 2.36 (t, 2H), 3.34 (t, 1H, $J = 9.3$, H-4), 3.51 (dd, 1H, $J = 9.2$, 3.8, H-2), 3.56 (dq, 1H, $J = 7$, 9.9, $CH_3C\underline{H}_2O$), 3.75 (m, 1H), 3.77 (m, 1H, H-5), 3.78 (dq, 1H, $J = 7.0$, 9.9, $CH_3C\underline{H}_2O$), 4.28 (dd, 1H, $J = 2.2$, 12.2, H-6b), 4.45 (dd, 1H, $J = 4.9$, 12.2, H-6a), 4.88 (d, 1H, $J = 3.9$, H-1)	14.1, 15.0, 22.7, 25.0, 29.2, 29.4 (2C), 29.5, 29.7 (2C), 32.0, 34.2, 63.6, 63.9, 70.0, 70.6, 72.2, 74.5, 98.3, 174.2
α-**3c**	0.89 (t, 3H), 1.29 (m, 19H), 1.61 (m, 2H), 2.38 (t, 2H), 3.37 (t, 1H, $J = 9$, H-4), 3.37 (t, 1H, $J = 9$), 3.47 (ddd, 1H, $J = 9.0$, 5.0, 3.0, H-5), 3.55 (t, 1H, $J = 9.0$), H-3), 3.62 (dq, 1H, $J = 7.0$, 9.5, $CH_3C\underline{H}_2O$), 3.94 (dq, 1H, $J = 7.0$, 9.5, $CH_3C\underline{H}_2O$), 4.28 (d, 1H, $J = 7.8$, H-1), 4.33 (d, 1H, $J = 5.0$, 12.0, H-6b), 4.38 (dd, 1H, $J = 3.0$, 12.0, H-6a]	14.0, 15.2, 22.7, 25.0, 29.2 (2C), 29.3, 29.4 (2C), 29.5, 31.9, 34.3, 63.7, 65.6, 70.6, 73.6, 74.0, 76.4, 102.5, 174.2

[a] Two-dimensional CH correlation NMR were used to confirm the assignment of the specta. NMR shifts for the homologous series of α- and β-glucoside esters, respectively, are well represented by the typical values given (except side chains). However, we noticed a clear concentration dependence in shifts and coupling constants for the H-6 protons.

Table 3. Mass Spectra Data of Alkyl Esters of Glucopyranosides **3** Prepared

Product	Exact Mass MH^+		MS m/z (%)
	calc.	found	
α-**3a**	335.2070	335.2075	335 (15), 289 (70), 127 (75), 57 (100)
α-**3b**	363.2383	363.2392	363 (10), 317 (75), 155 (70), 57 (60), 43 (100)
α-**3c**	391.2696	391.2681	391 (5), 345 (75), 183 (60), 57 (75), 43 (100)
α-**3d**	419.3009	419.3008	373 (25), 211 (25), 57 (60), 55 (70), 43 (100)
α-**3e**	447.3322	447.3314	447 (7), 401 (25), 185 (40), 93 (100)
α-**3f**	475.3635	475.3648	475 (2), 429 (10), 185 (30), 93 (100)
β-**3a**	335.2070	335.2074	335 (15), 288 (65), 127 (80), 57 (100)
β-**3b**	363.2383	363.2378	363 (5), 317 (60), 155 (60), 57 (55), 43 (100)
β-**3c**	391.2696	391.2688	391 (10), 345 (20), 185 (80), 93 (100)
β-**3d**	419.3009	419.3006	373 (20), 211 (25), 57 (65), 55 (80), 43 (100)
β-**3e**	447.3322	447.3330	447 (1), 401 (2), 185 (70), 93 (100)
β-**3f**	475.3635	475.3637	475 (0.5), 429 (1), 185 (60), 93 (100)

enzyme was found superior to that of the enzymes derived from *Mucor miehei* and *Humicola* sp., both of which were active but less selective giving rise to some diesters (Table 4).

Table 4. Lipase Activity, Rate of Conversion and Diester Content in the Synthesis of Ethyl Dodecanoyl-D-glucopyranoside using Various Immobilized lipases[a]

Lipase from	Activity KLU/g[b]	Conversion After 24 h (%)	Diester[c] Yield (%)
Candida antarctica	26.0	92.9	2.1
C. antarctica comp-A	26.8	19.4	1.5
C. antarctica comp-B	27.3	93.9	2.4
Mucor miehei	30.0	95.9	13.7
Humicola sp.	35.1	97.8	36.2
Candida cylindracea	23.8	12.4	0.5
Pseudomonas sp.	36.1	1.5	–

[a] The conversion of ethyl D-glucopyranoside was conducted at 70 °C and 0.01 bar, using 2 equivalents of dodecanoic acid and 6% (w/w) of different immobilized lipases. The conversions were measured by HPLC (mol-%).

[b] KLU = *K*ilo *L*ipase *U*nit, a lipase unit based on hydrolysis of tribututyrin (μmol fatty acid liberated per minute). A detailed description of this method is available from Novo Nordisk A/S (AF 95/5).

[c] Diester = ethyl 2,6-di-*O*-dodecanoyl-D-glucopyranoside.

The regioselectivities found are contrary to the selectivities of the enzymes observed in acidolysis of triglycerides. Thus, Lipozyme® is known to be 1,3-specific in acidolysis of triglycerides while the *Candida antarctica* lipase is unspecific in this reaction. Obviously, there is no clear correlation between triglyceride hydrolysis and glucoside ester synthesis. We also observed a faster reaction with the longer fatty acids (C_{12}-C_{18}) compared with the shorter (C_8-C_{10})-acids. This seems to be consistent with the general preference of lipases for lipophilic substrates.[9] The lipases from *Pseudomonas cepacia* and *Candida cylindracea* showed a very low catalytic activity under these reaction conditions.

An important feature of the lipases with regard to their applications in the present process is their heat stability since the reactions were carried out in melted fatty acids. To ensure the best stability and the most economic use, the enzymes were immobilised on hydrophobic carriers. In this way the enzymes could be used several times in a batch process with only a minor loss of activity.

In view of our good results in the laboratory, the synthetic procedure was tested on a pilot-plant scale. the pilot-plant synthesis was performed on a 20 kg scale using a mixture of α and β ethyl D-glucopyranoside, prepared by direct glucosylation of glucose in alcohol in the presence of an ion-exchange resin, and the fatty acids as substrates in a solvent-free process. The reaction were catalysed by 5% w/w of immobilised lipase from *Candida antarctica* which lead to 85–90% yield of 6-*O*-monoester within 24 hours. The enzyme used in the reactions could be recycled several times with no noticeable loss of activity.

The compounds prepared were subject to several performance evaluations, both as additives to detergents and as emulsifiers in personal care products.[10] In view of their performance and the simplicity of their preparation we believe this new group of biocompatible nonionic surfactants to have a considerable potential.

^{1}H- and ^{13}C-NMR spectra were obtained using a Bruker AM 500 spectrometer with TMS as the internal reference standard. The positive FAB mass spectra were obtained with a Kratos MS 50 RF mass spectrometer/DS 90 data system, equipped with an Ion-Tech saddle field gun, using 9–10 kV xenon atoms. The exact mass measurements were obtained at a resolving power of 8000–11000 by peak matching with successive introduction of reference compound (a mixture of NaI, RbI and CaI) and sample. The samples, dissolved in DMSO, were added to glycerol acidified with trichloroacetic acid (0.1 M). Optical rotations were measured in $CHCl_3$ using a Perkin-Elmer 241 polarimeter. Melting points are uncorrected. HPLC-analyses were performed on a Shimadzu LC-4A instrument equipped with a refractive index detector. A SiO_2-NH_2 column was used, with 96% EtOH as eluent. Critical micelle concentrations were determined using a Krüss Tensiometer K 10. Preparative chromatography was performed on Merck 60 silica gel using a hexane/EtOAc/MeOH gradient as eluent. Short path distillation was performed with a Leybold KDL-4 distillation equipment.

Lipozyme®, a lipase from *Mucor miehei*, is available as a commercial preparation from Novo-Nordisk A/S. *Candida antarctica* deposited at Deutsche Sammlung für Microorganismen (DSM) unter deposit number DSM 3855, DSM 3908 and DSM 3909, *Humicola sp.* DSM 3819, DSM 4109, *Pseudomonas sp.* DSM 3959, were all experimental preparations from Novo-Nordisk A/S. *Candida cylindracea* lipase and almond β-glucosidase were obtained from Sigma Chemical Company in activities of 690 μ/mg and 6.76 μ/mg, respectively. The lipases were used as immobilised preparations on macroporous acrylic or phenolic resins.[8] The activities are given in Table 4.

Ethyl α-D-Glucopyranoside:[11]
Ethyl D-glucopyranoside (30 g) with an anomeric ratio of about 1 is dissolved in 0.05 M sodium acetate buffer (400 mL, pH = 4.5) at 30 °C. β-Glucosidase (50 mg) is added and the mixture stirred for 10 d. In order to remove all enzyme activity the mixture is heated to 80 °C for 10 minutes and then concentrated and purified by column chromatography. The product crystallizes on concentration to give 8.2 g (55% yield); mp 107–109 °C (Lit.[11] 113–114 °C).

^{1}H-NMR (D_2O): $\delta = 1.22$ (t, 1 H, $J = 7.1$ Hz), 3.38 (t, 1 H, $J = 9.7$ Hz), 3.53 (dd, 1 H, $J = 9.8$ Hz), 3.56 (dq, 1 H, $J = 10$ Hz), 3.68 (t, 1 H, $J = 9.7$ Hz), 3.68 (ddd, 1 H, $J = 52, 2.2$ Hz), 3.74 (dd, 1 H, $J = 12.2$ Hz), 3.78 (dq, 1 H, $J = 10$ Hz), 3.88 (dd, 1 H, $J = 12.2$ Hz), 4.91 (d, 1 H, $J = 3.9$ Hz).

^{13}C-NMR ($CDCl_3$): $\delta = 15.0$, 61.5, 64.8, 70.6, 72.2, 72.6, 74.1, 98.8.

Ethyl β-D-Glucopyranoside:[12]
Ethyl 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranoside:
To a suspension of silver carbonate (30 g, 0.11 mol) in EtOH 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (41.1 g, 0.10 mol) is added in small portions over a period of 40 min. The mixture is stirred for 15 h, diluted with CH_2Cl_2 (40 mL) and filtrated through celite and activated carbon. Ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside crystallizes on concentration; yield: 23.4 g (62%).

Ethyl β-D-Glucopyranoside:
The ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside is deacetylated with 1 M NaOMe (2 mL) in MeOH (80 mL) in 20 h at r.t.. The mixture is neutralized with Amberlite IR-120 and concentrated to give a quantitative yield of the product as a foam.

^{1}H-NMR (D_2O): $\delta = 1.22$ (t, 1 H, $J = 7.1$ Hz), 3.24 (dd, 1 H, $J = 9.4$ Hz), 3.36 (t, 1 H, $J = 9.4$ Hz), 3.44 (ddd, 1 H, $J = 6.6, 2.1$ Hz), 3.47 (t, 1 H, $J = 9.1$ Hz), 3.76 (dd, 1 H, $J = 12.3$ Hz), 3.71 (dq, 1 H, $J = 9.9$ Hz), 3.90 (dd, 1 H, $J = 12.3$ Hz), 3.96 (dq, 1 H, $J = 9.9$ Hz), 4.45 (d, 1 H, $J = 8.0$ Hz).

^{13}C-NMR ($CDCl_3$): $\delta = 15.2$, 61.7, 67.1, 70.6, 74.1, 76.8 (2C), 102.8.

Enzymatic Synthesis of Ethyl β-D-Glucopyranoside:
Almond β-glucosidase (100 mg) is added to a solution of D-glucose (2 g) in 85% EtOH (100 mL). After stirring at r.t. for 14 d, HPLC shows about 70% conversion. The enzyme is deactivated by heating the reaction mixture to 70 °C for 1 h. Precipitated solids are removed by filtration and the solvent is evaporated under reduced pressure. The product is dissolved in water (100 mL) and excess glucose is fermented by adding bakers' yeast. After 3 d, HPLC analysis shows total conversion of glucose and the yeast is removed by filtration through celite and the solvent evaporated to give the product; yield: 42% (0.97 g). The spectroscopic data corresponds to those given above.

Ethyl 6-*O*-Alkanoyl-D-glucopyranosides; General Procedure:
Ethyl α-D-glucopyranoside (2.0 g, 9 mmol) is suspended in melted fatty acid (2 eq) at 70 °C. Lipozyme® (0.33 g) is added and the mixture stirred under reduced pressure until HPLC-analysis shows that the reaction is complete. The mixture is diluted with acetone (50 mL) filtered and concentrated. The product is purified by chromatography (Table 1, 2 and 3).

Ethyl 6-*O*-Alkanoyl-β-D-glucopyranosides:
Following the general procedure for ethyl 6-*O*-alkanoyl-α-D-glucopyranosides starting from ethyl β-D-glucopyranoside (3.0 g, 14 mmol), fatty acid (2 equiv) and Lipozyme® (0.5 g) (Table 1, 2 and 3).

Large-Scale Synthesis of Alkyl 6-*O*-Alkanoyl-D-glucopyranosides; General Procedure:
Glucose (8 kg, 44 mol) is suspended in abs. EtOH (25 L, 428 mol). Ion exchange resin (Amberlyst 15, 1.6 kg) is added and the reaction mixture is refluxed under efficient mechanical stirring for 18 h. The ion exchange resin is removed by filtration and excess EtOH is distilled off under reduced pressure, yielding crude ethyl D-glucopyranoside as a viscous syrup (about a 1:1 mixture of the α- and the β-anomers, containing about 4% unconverted glucose). Melted coconut fatty acids (12.7 kg, 57 mol) are added and the mixture is heated under stirring to 70 °C. Immobilised *Candida antarctica* lipase (400 g) is added and the stirring is continued at 70 °C under reduced pressure (0.01 bar) until a > 90% conversion is determined by HPLC (reached in 28 h). The enzyme is removed by filtration (at 70 °C, under pressure) yielding a crude product (21.3 kg) consisting of 70 wt % of 6-*O*-monoester, 3% diester, 21% fatty acid and 7% ethyl D-glucoside. The excess of fatty acid is removed by short-path distillation (at 130 °C and 0.004 mbar) resulting in a product of > 85 wt % monoester. The monoester obtained above consists of > 96% 6-*O*-ester of ethyl D-glucopyranoside, the rest being 6-*O*-monoester of D-glucose.

If glucose monohydrate and 96% EtOH are used in the above procedure, the crude ethyl D-glucopyranoside contains about 15% unconverted glucose, resulting in a product with a content of > 10% glucose ester in the monoester fraction.

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(1) Kahn, R. *Adv. Carbohydr. Chem. and Biochem.*, **1976**, *33*, 271.
Baumann, H.; Bühler, M.; Fochem, H.; Hirsinger, F.; Zoebelein, H.; Falbe, J. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 41.

(2) Reinefeld, E.; Korn, H.-F. *Die Stärke*, **1968**, *20*, 181.
Yoshimoto, K.; Tahara, K.; Suzuki, S.; Sasaki, K.; Nishikawa, Y.; Tsuda, Y. *Chem. Pharm. Bull.* **1979**, *27*, 2661.
Albano-Garcia, E.; Lorica, E.G.; Pama, M.; de Leon, L. *Philipp. J. Coconut Stud.* **1980**, *5*, 51.
Plusquellec, D.; Baczko, K. *Tetrahedron Lett.* **1987**, *28*, 41.
Myhre, D.V. *US Patent* 3597417 (1971).
Hennen, W.J.; Sweers, H.M.; Wang, Y.-F.; Wong, C.-H. *J. Org. Chem.* **1988**, *53*, 4939.

(3) Sweers, H.M.; Wong, C.-H. *J. Am. Chem. Soc.* **1986**, *108*, 6421.
Chopineau, J.; McCafferty, F.D.; Therisod, M.; Klibanov, A.M. *Biotechnol. Bioeng.* **1988**, *31*, 208.
Shaw, J-F.; Klibanov, A.M. *Biotechnol. Bioeng.* **1987**, *29*, 648.
Therisod, M.; Klibanov, A.M. *J. Am. Chem. Soc.* **1987**, *109*, 3977.
Kloosterman, M.; Mosmuller, E.W.J.; Schoemaker, H.E.; Meijer, E.M. *Tetrahedron Lett.* **1987**, *28*, 2989.

(4) Therisod, M.; Klibanov, A.M. *J. Am. Chem. Soc.* **1986**, *108*, 5638.

(5) Riva, S.; Chopineau, J.; Kieboom, A.P.G.; Klibanov, A.M. *J. Am. Chem. Soc.* **1988**, *110*, 584.

(6) Seino, H.; Uchibori, T.; Nishitani, T.; Inamasu, S. *J. Am. Oil Chem. Soc.* **1984**, *61*, 1761.

(7) For a preliminary communication see Björkling, F.; Godtfredsen, S.E.; Kirk, O. *J. Chem. Soc. Chem. Commun.* **1989**, 934.

(8) Heldt-Hansen, H.P.; Ishii, M.; Patkar, A.S.; Hansen, T.T.; Eigtved, P. *Biocatalysis in Agricultural Biotechnology*, ACS Symposium series *389*, chapter 11, 158, Washington DC, 1989.
Danish Patent Appl. No 3250/88.

(9) Macrae, A.R.; Hammond, R.C. *Biotech. Genetic Engineer. Rev.* **1985**, *3*, 193.
Miller, C.; Austin, H.; Posorske, L.; Gonzlez, J. *J. Am. Oil Chem. Soc.* **1988**, *65*, 927.

(10) Will be reported elsewhere.

(11) Fischer, E. *Ber. Dtsch. Chem. Ges.* **1895**, *28*, 1145.

(12) Koenigs, W.; Knorr, E. *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 957.