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Candida antarctica Lipase B Catalyzes the Regioselective Esterification of Ecdysteroids at the C-2 OH

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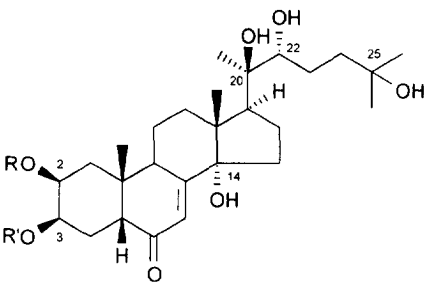
Abstract: Immobilized *Candida antarctica* lipase B (Novozym 435) catalyzes the regioselective acylation of 20R-hydroxyecdysone **(1a)** and its congeners **2a**, **3a** and **4a** at the C-2 OH in high yield and purity. © 1997 Elsevier Science Ltd.

INTRODUCTION

Ecdysteroids are a group of tetracyclic polyhydroxylated compounds present in exceedingly small amounts in most classes of invertebrates. In insects, crustaceans and other arthropods they play a fundamental role in controlling many important physiological functions related to the development (moulting and metamorphosis), reproduction, metabolism, excretion and others [1]. Ecdysteroids are also widely spread in the vegetal kingdom and this has resulted in the isolation of a large number of these compounds in substantial amount, thus allowing extensive studies on chemical behaviour, biological activity, biosynthesis and metabolism.

A number of ester derivatives of ecdysteroids have been found in nature. For example, the 20*R*-hydroxyecdysone **(1a)** (20-OH-ECD), the most abundant and most frequently isolated of these compounds, has been shown to occur in plants as the 2-acetate **1b**,[2] 2-cinnamate **1c**,[3] 3-*p*-coumarate,[3] 22-acetate,[2] 25-acetate (viticosterone)[4] and 2,22-diacetate.[2] The 3-acetate **1d**,[5] the 22-acetate[6] and the 22-esters with linoleic, palmitic, oleic and stearic acids[7] have been found in animals and shown to be deactivated forms of the moulting hormone.

The presence of many OH's in the ecdysteroids molecule poses the problem of selective formation of esters at specific positions of their skeleton. Studies on the chemical acetylation of **1a**,[8] indicated that, among secondary OH's, the C-2 OH was much more reactive than the C-22 OH, which in turn reacted faster than C-3 OH. As expected, the hindered tertiary OH's were less reactive, the C-25 OH reacting slower that C-3 OH, while C-14 OH and C-20 OH were almost non reactive. Notwithstanding, attempts to acetylate **1a** under controlled conditions to furnish the 2-acetate **1b**, always resulted in a mixture of esters in which the desired compound was predominant but could be isolated only after careful separations.



1a R = R' = H
1b R = Ac; R' = H
1c R = PhCH=CH-CO; R' = H
1d R = H; R' = Ac
1e R = CH_3-$(CH_2)_{10}$-CO; R' = H
1f R = CH_3-$(CH_2)_{14}$-CO; R' = H
1g R = $PhCH_2$-O_2C-CH_2-CO; R' = H
1h R = HO_2C-CH_2-CO; R' = H
1i R = H; R' = PhCH=CH-CO

Esterification at specific position of **1a** has been possible by making recourse to a protection-deprotection procedure exploiting the presence of two 1,2-diol systems at positions 2,3 and 20,22.[8] Thus the 2,3-acetonide and the 20,22-acetonide of **1a**, easily formed by modulating the ketalization of **1a** with acetone in the presence of acid catalysts, have been esterified at the C-22 OH and C-2 OH respectively, and the 2-acetate as well as many 22-esters of **1a** have been obtained after acid hydrolysis of the acetonide group.

In a recent paper on the partial synthesis of some minor ecdysteroids and analogues,[9] the selective acetylation of **1a** has been revisited in detail. However, difficulties have been encountered in obtaining the pure 2-acetate **1b**, either by direct acetylation or by hydrolysis of the acetonide moiety of the 20,22-monoacetonide-2-acetate. In both cases the ^{1}H-NMR spectrum clearly indicated that the desired compound **1b** was always contaminated by ca. 30% of the isomeric 3-acetate **1d** which co-migrates on TLC.

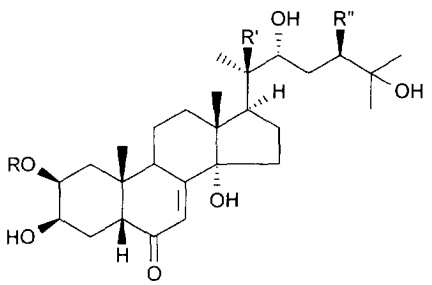
In previous papers we have developed a methodology for the regioselective esterification of polyhydroxy compounds by means of activated esters under the catalysis of enzymes in anhydrous organic solvents.[10] By applying this methodology to ecdysteroids we have found that the lipase B from *Candida antarctica* supported on acrylic resin (commercialized under the trade name of Novozym 435) was able to discriminate between the alcoholic substituents of these substrates furnishing the esters at the C-2 OH in high to good yield, without the appreciable formation of esters in other hydroxylated positions. In the following we report the details of this research.

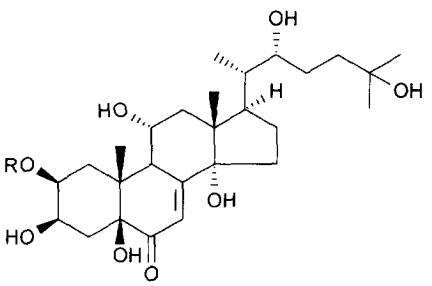
RESULTS AND DISCUSSION

Due to their highly hydroxylated nature, ecdysteroids are soluble only in polar solvents. A first attempt to acylate 20-OH-ECD **1a** in pyridine solution with subtilisin (protease Carlsberg), an enzyme which is known to be at least partially active in such a solvent, was completely unsuccessful. The starting material was recovered almost unreacted or partly converted into a mixture of acyl derivatives.

Looking for more suitable reaction conditions, after experimentation we found that a ca 20 mM solution of **1a** in *t*-AmOH containing 10% pyridine and an excess of vinylacetate was nicely converted by Novozym 435 into a single reaction product. Filtration of the enzyme, evaporation of the solvent and crystallization from AcOEt/cyclohexane allowed the isolation of the reaction product in 70% yield. This compound was recognized as the 20-OH-ECD 2-acetate **1b** on the basis of its spectroscopic data. The FAB-MS negative spectrum contained a $(M-H)^-$ ion at m/z 521 indicating the presence of an additional acetyl group in the molecule. The ^{1}H-NMR spectrum showed, in addition to a singlet at δ 2.02 (s, 3H) for an acetate group, a low-field ddd for an acetylated oxymethine at δ 4.84 (J = 11.5, 4.0, 3.0 Hz), and a broad singlet for an oxymethine at δ 3.97 ($W_{1/2}$ = 8 Hz). On the basis of multiplicity and J values, these signals were attributed to H-2 and H-3, respectively. No traces of the signals of the isomeric acetates, and in particular of the 3-acetate **1d**, could be detected.

Structurally analogous ecdysteroids showed a similar behaviour. Thus, ecdysone **2a**, makisterone **3a** and the highly oxygenated muristerone A **4a**[11] furnished the corresponding 2-acetates **2b**, **3b**, **4b** in excellent to good isolated yields.





2a R = R' = R" = H
2b R = Ac; R' = R" = H
3a R = H; R' = OH; R" = Me
3b R = Ac; R' = OH; R" = Me

4a R = H
4b R = Ac

The reaction could then be extended to the introduction of long-chain acid residues. In fact by reacting **1a** with vinyl laurate or trifluoroethyl palmitate in the presence of Novozym 435, the corresponding 20-OH-ECD 2-laurate **1e** and 20-OH-ECD 2-palmitate **1f** were obtained, even though in moderate yield due to lower conversions.

With this information in hand, we decided to synthesize the 20-OH-ECD 2-cinnamate **1c**, isolated from the bark of *Dacrydium intermedium*.[3] 2,2,2-Trifluoroethylcinnamate was used as an acylating reagent under the usual conditions, but, disappointingly, **1a** was found to be completely unreactive even after a prolonged reaction time and in the presence of an excess of enzyme and activated ester. A plausible esplanation for this behaviour emerged from a comparison with the facile and complete cinnamoylation of BuOH under the same conditions. This indicated that the activated cinnamoyl ester interacted with the enzyme to form the corresponding acyl-enzyme intermediate. However, very likely, the bulkiness and the rigidity of the acyl residue encumbered the active site, preventing the approach by a large nucleophile such as **1a**.

To overcome this failure, we applied to **1a** the strategy we have developed earlier for the synthesis of the 6"-cinnamate of the flavonoid glucoside isoquercitrine.[12] This strategy consists in a combined approach based on the enzymatic introduction of a small malonate unit which is then chemically elaborated into the desired cinnamate by reaction with benzaldehyde (Knoevenagel reaction).

To this end, dibenzylmalonate was reacted with 20-OH-ECD **1a** in the presence of Novozym 435 and the 2-malonate benzyl ester **1g** was formed in 62% isolated yield. The ^{1}H-NMR spectrum of **1g** contained the signals of H-2 at δ 5.04 (ddd, J = 12.5, 4.0, 3.0 Hz), H-3 at δ 4.12 (bs) and the resonances for the methylene protons of the malonate benzyl ester moiety at δ 5.18 and 3.49 and 3.40 (AB system, J = 16 Hz). However, at variance with the acetylation, trace signals (< 3%) due to the isomeric 3-malonate benzyl ester, superimposed in TLC, could be detected at δ 5.32 (bs, H-3) and 3.96 (m, H-2).

Catalytic hydrogenation of **1g** on 5% Pd/C in THF solution afforded 20-OH-ECD 2-malonate **1h** in quantitative yield after catalyst filtration and solvent evaporation at room temperature. The crude malonate was directly used in the subsequent reaction with benzaldehyde in anhydrous pyridine at 60 °C to give the condensation product in quantitative yield. To our great surprise, the ^{1}H-NMR spectrum of this product clearly showed the presence of the signals due to two compounds, the 2-cinnamate **1c** and the 3-cinnamate **1i** in 7:3 ratio. In fact, signals at δ 5.00 (ddd, J = 11.5, 4.0, 3.0 Hz) and 4.08 (q, J = 3.0 Hz) were found for H-2 and H-3 of the former compound, and resonances at δ 5.18 (bs, $W_{1/2}$ = 8 Hz) and 3.90 (ddd, J = 11.0, 4.0, 3.0 Hz) for H-3 and H-2 of the latter, respectively. As the 2-esters of **1a** are stable in solution even at 60 °C, we believe that the 3-cinnamate is formed by acyl migration during the condensation with benzaldehyde which takes place under basic conditions. Migration of acyl groups between vicinal *cis*-

hydroxy groups is known to be favoured under acid[13] and basic catalysis and has been previously reported during derivatization of ecdysteroids.[14]

CONCLUSIONS

Candida antarctica lipase B (Novozym 435) has been shown to an excellent catalyst for the regioselective acylation of ecdysteroids with short and long chain activated esters to afford the corresponding 2-esters in one step and in good yields. This is at variance with chemical protocols which usually affords the 2-esters contaminated by a substantial amount of the co-eluting 3-esters.

However, as shown, the enzymatic methodology has a limitation when applied to these specific substrates. Bulky acyl groups such as the cinnamoyl moiety, encumbers the active site of this lipase and inhibits the access of the nucleophile, thus preventing the reaction to take place. The failure to directly introduce a cinnamoyl group was circumvented by a combination of enzymatic and chemical reactions, emulating a Knoevenagel condensation. Whereas the enzymatically formed 2-malonate benzyl ester **1g** contained only traces of the isomeric 3-malonate benzyl ester, the final cinnamate was a 7:3 mixture of the 2-cinnamate and the 3-cinnamate, due to acyl migration under the basic conditions employed in the condensation reaction.

Despite this inconvenience, this report further enlightens the versatility and efficiency of Novozym 435 for the selective acylation of complex polyhydroxylated molecules[15] and specifically for the obtainment of pure 2-esters of ecdysteroids.

EXPERIMENTAL

Materials and Methods. Lipase B from *Candida antarctica* supported on acrylic resin (Novozym 435) was a generous gift from Novo-Nordisk. ^{1}H-NMR spectra were recorded on a Bruker 300 AC instrument operating at 300 MHz. FAB-MS spectra in the negative mode (FAB-) were obtained on a VG 7070 EQ-HF instrument equipped with its own source, operating at 8 keV with Xe gas and in diethanolamine as a matrix. Flash column chromatography (FC) and TLC were carried out using Merck silica gel 60 (70-230 mesh) and precoated silica gel 60 F_{254} plates, respectively. Spot on TLC were visualized by fluorescence quenching at 254 nm and by spraying with anisaldehyde-H_2SO_4 reagent (Komarowsky's reagent) followed by heating.

General Procedure for Acylation of Ecdysteroids. The ecdysteroid (0.104 mmol) was dissolved into 5 ml of *tert*-amyl alcohol containing 10% pyridine and the solution treated with an excess (10 - 15 eq) of activated ester (vinyl acetate, vinyl laurate, trifluoroethyl palmitate, dibenzyl malonate). Novozym 435 (100 mg) was added and the suspension shaken at 45 °C, following the reaction by TLC (eluent $CHCl_3$:MeOH = 8:2 or 9:1). After the appropriate time, the enzyme was filtered off, the solvent evaporated and the reaction product recovered.

20-OH-ECDYSONE 2-ACETATE (1b). Obtained by reaction of **1a** with vinyl acetate (10 eq) after 7 days. The crude residue was crystallized from EtOAc/cyclohexane to give 90% yield of **1b**, mp 220-223 °C (lit. [8] 219-220 °C). Rf = 0.6 ($CHCl_3$:MeOH = 8:2); FAB$^-$ *m/z* 521 $(M-H)^-$; ^{1}H-NMR (DMSO-d_6, 60 °C) δ 5.68 (1H, d, J = 2.0 Hz, H-7), 4.83 (1H, ddd, J = 11.5, 4.0, 3.0 Hz, H-2), 3.97 (1H, bq, J = 3.0 Hz, H-3), 3.18 (1H, dd, J = 9.5, 1.5 Hz, H-22), 3.11 (1H, ddd, J = 11.5, 7.0, 2.0 Hz, H-5), 2.02 (3H, s, CH_3CO), 1.10 and 1.08 (3H and 6H, s, 21-, 26-, 27-Me), 0.89 (3H, s, 19-Me), 0.80 (3H, s, 18-Me); Anal. Calcd. for $C_{29}H_{46}O_8 \cdot 1.5\ H_2O$: C, 63.53; H, 8.99. Found: C, 63.18; H, 9.03.

20-OH-ECDYSONE 2-MALONATE BENZYL ESTER (1g). Obtained by reaction of **1a** with dibenzyl malonate (15 eq) after 7 days. Purification by FC ($CHCl_3$:MeOH = 9:1) gave **1g** in 62% yield, mp 158-160 °C (from EtOAc/hexane). Rf = 0.59 ($CHCl_3$:MeOH = 8:2); FAB$^-$ *m/z* 655 $(M-H)^-$; ^{1}H-NMR ($CDCl_3$) δ 7.4-7.3 (5H, m, Ph), 5.84 (1H, d, J = 2.0 Hz, H-7), 5.18 (2H, s, $PhCH_2OCO$), 5.04 (1H, ddd, J = 12.5, 4.0, 3.0 Hz, H-2), 4.12 (1H, m, H-3), 3.49 and 3.40 (each 1H, d, J =16 Hz, AB system of $COCH_2COO$), 3.45 (1H, dd, J = 9.5, 1.5 Hz, H-22), 3.06 (1H, ddd, J = 11.5, 7.0, 2.5 Hz, H-5), 1.25 and 1.21 (6H and 3H, s, 21-, 26-, 27-Me), 0.98 (3H, s, 19-Me), 0.88 (3H, s, 18-Me). Trace signals at: δ 5.32 (bs, H-3), 5.20 (s, $PhCH_2O$), 3.96 (m, H-2) indicate the presence (< 3%) of the isomeric 3-malonate benzyl ester. Anal. Calcd. for $C_{37}H_{52}O_{10} \cdot H_2O$: C, 65.84; H, 8.07. Found: C, 65.01; H, 8.68.

20-OH-ECDYSONE 2-CINNAMATE (1c) and 20-OH-ECDYSONE 2-CINNAMATE (1i). a) A solution of 20-OH-ecdysone 2-malonate benzyl ester **1g** (60 mg, 0.09 mmol) in 5 ml anhydrus THF with a catalytic amount of Pd/C (5%) was stirred for 3.5 h under H_2. The catalyst was filtered and solvent removed under vacuum at room temperature to afford the 2-malonyl ecdysone **1h** in quantitative yield.

b) 20-OH-ecdysone 2-malonate **(1h)** (41 mg, 0.072 mmol) was dissolved in 3 ml of dry pyridine and then molecular sieves 4Å, benzaldehyde (3 eq) and piperidine (10μl) were added. The suspension was stirred at 60 °C under N_2 for 1 h, and after filtration and solvent evaporation 39 mg (89 % yield) of a 7:3 mixture of **1c** and **1i** was obtained. Rf = 0.59 ($CHCl_3$:MeOH = 8:2); FAB$^-$ *m/z* 609 $(M-H)^-$; ^{1}H-NMR (DMSO-d_6, 80 °C): **1c** δ 7.70-7.68 (2H, m, Ph), 7.48-7.42 3H, m, Ph), 7.71 (1H, d, J = 16.0 Hz, CH=CH-CO), 6.58 (1H, d, J = 16.0 Hz, CH=CH-CO), 5.71 (1H, d, J = 2.0 Hz, H-7), 5.00 (1H, ddd, J = 11.5, 4.0, 3.0 Hz, H-2), 4.08 (1H, q, J = 3.0 Hz, H-3), 1.11 (9H, s, 21-, 26-, 27-Me), 0.92 (3H, s, 19-Me), 0.81 (3H, s, 18-Me); **1i** δ 6.68 (1H, d, J = 16.0 Hz, CH=CH-CO), 6.63 (1H, d, J = 16.0 Hz, CH=CH-CO), 5.18 (1H, bs, $W_{1/2}$ = 8, H-3), 3.90 (1H, ddd, J = 11.5, 4.0, 3.0 Hz, H-2). Anal. Calcd. for $C_{36}H_{50}O_8 \cdot H_2O$: C, 68.75; H, 8.34. Found: C, 68.94; H, 8.54.

20-OH-ECDYSONE 2-LAURATE (1e). Obtained by reaction of **1a** with vinyl laurate (10 eq) in 69% yield after 6 days (FC, $CHCl_3$:MeOH = 92:8), as an amorphous solid, mp 78-80 °C. Rf = 0.5 ($CHCl_3$:MeOH = 85:15); FAB$^-$ *m/z* 661 $(M - H)^-$; ^{1}H-NMR (DMSO-d_6, 60 °C) δ 5.68 (1H, d, J = 2.0 Hz, H-7), 4.87 (1H, ddd, J = 11.5, 4.0 and 3.0 Hz, H-2), 3.97 (1H, m, H-3), 3.20 (1H, dd, J = 9.5, 1.5 Hz, H-22), 3.13 (1H, ddd, J = 11.5, 7.0, 2.0 Hz, H-5), 2.29 (2H, t, J = 7.0 Hz, CH_2CH_2COO), 1.11 (9H, s, 21-, 26-, 27-Me), 0.91 (3H, s, 19-Me), 0.87 (3H, t, J = 5.5 Hz, $CH_3CH_2CH_2$), 0.81 (3H, s, 18-Me). Anal. Calcd. for $C_{39}H_{66}0_8 \cdot H_2O$: C, 68.77; H, 10.07. Found: C, 68.56; H, 10.15.

20-OH-ECDYSONE 2-PALMITATE (1f). Obtained by reaction of **1a** with trifluoroethyl palmitate (10 eq) in 50% yield after 6 days (FC, $CHCl_3$:MeOH = 95:5), as an amorphous solid, mp 87-89 °C. Rf = 0.3 ($CHCl_3$:MeOH = 9:1); FAB$^-$ *m/z* 689 $(M-H)^-$; ^{1}H-NMR (DMSO-d_6, 60 °C) δ 5.68 (1H, d, J = 2.0 Hz, H-7), 4.86 (1H, ddd, J = 11.5, 4.0, 3.0

Hz, H-2), 3.96 (1H, m, H-3), 3.18 (1H, dd, J = 9.5, 1.5 Hz, H-22), 3.11 (1H, ddd, J = 11.5, 7.0, 2.0 Hz, H-5), 2.29 (2H, t, J = 7.0 Hz, $CH_2\underline{CH_2}COO$), 1.12 and 1.10 (3H and 6H, s, 21-, 26-, 27-Me), 0.91 (3H, s, 19-Me), 0.88 (3H, t, J = 5.5 Hz, $\underline{CH_3}CH_2CH_2$), 0.80 (3H, s, 18-Me). Anal. Calcd. for $C_{41}H_{70}O_8$: C, 71.25; H, 10.22. Found: C, 70.74; H, 10.31.

ECDYSONE 2-ACETATE (2b): Obtained by reaction of **2a** with vinyl acetate (10 eq) in 95% yield after 2 days (FC, $CHCl_3$:MeOH = 9:1), mp = 213-216 °C (from AcOEt/cyclohexane). Rf = 0.58 ($CHCl_3$:MeOH = 8:2); FAB^- *m/z* 505 $(M-H)^-$; ^{1}H-NMR (DMSO-d_6, 60 °C) δ 5.68 (1H, d, J = 2.0 Hz, H-7), 4.84 (1H, ddd, J = 11.5, 4.0, 3.0 Hz, H-2), 3.97 (1H, m, $W_{1/2}$ = 8, H-3), 3.44 (1H, bdd, J = 9.0, 2.5 Hz, H-22), 3.12 (1H, ddd, J = 11.5, 7.0, 2.0 Hz, H-5), 2.01 (3H, s, $C\underline{H_3}CO$), 1.12 and 1.10 (each 3H, s, 26-, 27-Me), 0.91 (3H, s, 19-Me), 0.88 (3H, d, J = 7.0 Hz, 21-Me), 0.62 (3H, s, 18-Me); Anal. Calcd. for $C_{29}H_{46}O_7 \cdot H_2O$: C, 66.37; H, 9.23. Found: C, 66.01; H, 9.83.

MAKISTERONE 2-ACETATE (3b). Obtained by reaction of **3a** with vinyl acetate (10 eq) in 76.5% yield after 7 days (FC, $CHCl_3$:MeOH = 9:1), mp = 214-217 °C (from AcOEt/cyclohexane). Rf = 0.7 ($CHCl_3$:MeOH = 8:2); FAB^- *m/z* 535 $(M-H)^-$; ^{1}H-NMR (DMSO-d_6, 60 °C) δ 5.68 (1H, d, J = 2.0 Hz, H-7), 4.82 (1H, ddd, J = 12.0, 4.0, 3.0 Hz, H-2), 3.98 (1H, q, J = 3.0 Hz, H-3), 3.30 (1H, bd, J = 10.0 Hz, H-22), 3.10 (1H, ddd, J = 11.5, 7.0, 2.0 Hz, H-5), 2.02 (3H, s, $C\underline{H_3}CO$), 1.10, 1.08 and 1.07 (each 3H, s, 21-, 26-, 27-Me), 0.91 (3H, s, 19-Me), 0.88 (3H, d, J = 7.0 Hz, 28-Me), 0.82 (3H, s, 18-Me). Anal. Calcd. for $C_{30}H_{48}O_8 \cdot 1.5H_2O$: C, 63.90; H, 9.12. Found: C, 63.02; H, 9.73.

MURISTERONE A 2-ACETATE (4b). Obtained by reaction of **4a** with vinyl acetate (10 eq) in 63% yield after 7 days (FC, $CHCl_3$:MeOH = 97:3), as a foam. Rf = 0.36 ($CHCl_3$:MeOH = 9:1); FAB^- *m/z* 551 $(M-H)^-$; ^{1}H-NMR (DMSO-d_6, 60 °C) δ 5.74 (1H, d, J = 2.0 Hz, H-7), 5.14 (1H, ddd, J = 12.0, 4.0, 3.0 Hz, H-2), 4.11 (1H, q, J = 3.0 Hz, H-3), 3.98 (1H, td, J = 9.0, 3.0 Hz, H-11), 3.30 (1H, bd, J = 10.0 Hz, H-22), 2.01 (3H, s, $C\underline{H_3}CO$), 1.10, 0.90 and 0.89 (each 3H, s, 21-, 26-, 27-Me), 0.95 (3H, s, 19-Me), 0.80 (3H, s, 18-Me). Anal. Calcd. for $C_{29}H_{46}O_9 \cdot 2H_2O$: C, 60.63; H, 8.78. Found: C, 60.03; H, 9.01.

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