Journal of Medicinal Chemistry

Volume 45, Number 17 August 15, 2002

Letters

6α-Ethyl-Chenodeoxycholic Acid (6-ECDCA), a Potent and Selective FXR Agonist Endowed with Anticholestatic Activity

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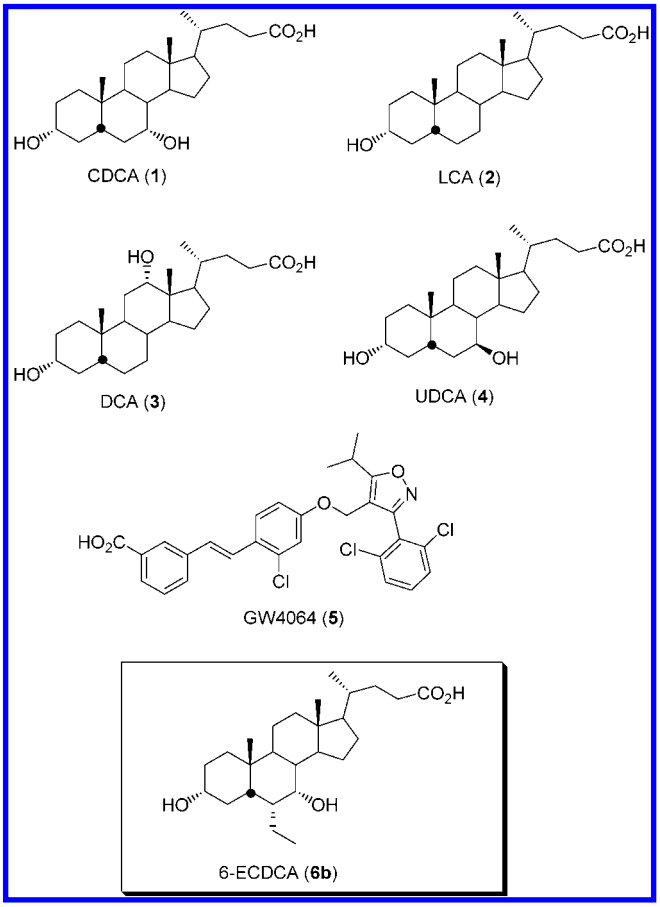
Received April 11, 2002

Abstract: A series of 6α-alkyl-substituted analogues of chenodeoxycholic acid (CDCA) were synthesized and evaluated as potential farnesoid X receptor (FXR) ligands. Among them, 6α-ethyl-chenodeoxycholic acid (6-ECDCA) was shown to be a very potent and selective FXR agonist (EC_{50} = 99 nM) and to be endowed with anticholeretic activity in an in vivo rat model of cholestasis.

Introduction. The farnesoid X receptor (FXR, *NRIH4*) is an orphan member of the superfamily of nuclear receptors, a growing ensemble of ligand-dependent transcription factors that are activated by small, lipophilic hormones.[1] FXR was originally proposed to be a receptor for the intermediary metabolite farnesol.[2] However, farnesol does not bind to FXR and supraphysiological concentrations were required to activate the receptor. In 1999, three groups independently proposed FXR as a nuclear receptor for bile acids (BA).[3]

Chart 1



These studies revealed that physiological concentrations of BA bound and activated FXR, with the primary bile acid chenodeoxycholic (CDCA, **1**, Chart 1) being the most potent ligand with EC_{50} values ranging between 10 and 50 μM.[3] Lithocholic (LCA, **2**) and deoxycholic (DCA, **3**) acid also activate FXR, while ursodeoxycholic acid (UDCA, **4**) is inactive. Nonsteroid ligands for FXR have also been discovered, of which the combinatorial chemistry-derived isoxazole GW4064 (**5**) is the most potent.[4]

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10.1021/jm025529g CCC: $22.00
Published on Web 07/20/2002

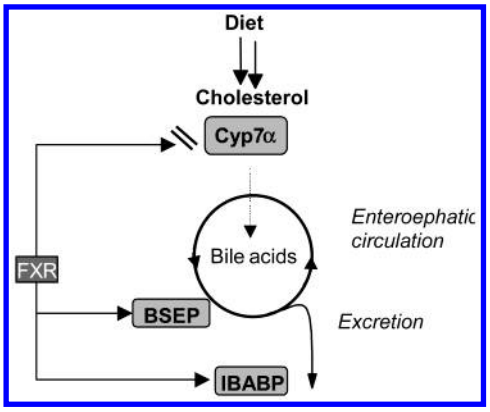


Figure 1. FXR regulates the expression of target genes involved in bile acids and cholesterol homeostasis.

FXR shares a common modular architecture with the other members of the nuclear receptor superfamily, comprising a highly conserved DNA binding domain (DBD) and a carboxy-terminal ligand binding domain (LBD). The DBD recognizes specific DNA sequences, known as response elements, within the enhancer region of the receptor's target genes. FXR binds as a heterodimer with the 9-*cis* retinoic acid receptor (RXR) to an IR-1 response element. The binding of a small, lipophilic ligand to the LBD results in the recruitment of transcriptional coactivators (e.g., SRC1), which couple the receptor to the cellular transcriptional machinery.[5]

In its capacity as a BA receptor, FXR regulates the expression of several target genes involved in the BA homeostasis (Figure 1). In particular, FXR indirectly represses the expression of the BA biosynthetic enzymes CYP7A and CYP8B by increasing the levels of the inhibitory nuclear receptor SHP in the liver and intestine.[6] BA-activated FXR also positively regulates the expression of genes encoding proteins involved in the transport of BA, including I-BABP, BSEP, and cMOAT.[7] Although many of the molecular mechanisms involved in FXR-mediated gene transcription regulation remain to be fully understood, a picture has emerged of the role of this receptor in BA and cholesterol homeostasis.[8] Another intriguing therapeutic opportunity associated with FXR modulation is the control of bile flow. Indeed, the FXR-mediated activation of SHP causes the negative feedback regulation of NTCP, the principal BA transporter in the liver. Notably, FXR null mice display defects in BA homeostasis such as elevated serum BA, reduced BA pools, and reduced fecal BA secretion.[9] So, FXR may coordinate an integrated pathway aimed at the down-regulation of BA import and synthesis. The regulation of this process may protect hepatocytes from BA toxicity.

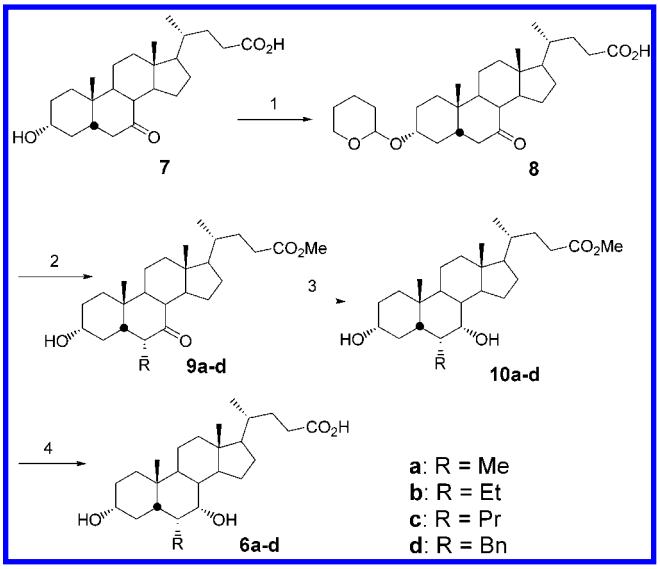
Cholestasis is a condition characterized by an impairment or cessation of bile flow that occurs in a variety of human liver diseases such as primary biliary cirrhosis, primary sclerosing cholangitis, cystic fibrosis, and intrahepatic cholestasis of pregnancy.[10] Currently, drug treatment for cholestasis is limited to the secondary BA UDCA (**4**)[11] whose long-term efficacy remains unproven. The molecular mechanism of action of UDCA (**4**) is unknown, although its anticholestatic activity may be due in part to the displacement of more hydrophobic BA from the hepatocyte. Given the proposed role of FXR in BA biosynthesis and transport from the liver, we sought to identify potent BA-based ligands. We decided to investigate a series of CDCA analogues as potential FXR ligands and explore their in use the treatment of cholestasis.

Table 1. Binding Potency of Synthetic Bile Acids to FXR

compd	EC_{50} (μM)	efficacy[a]
5 (GW4064)	0.037 ± 0.006	117 ± 3
1 (CDCA)	8.66 ± 0.45	100 ± 3
6a	0.75 ± 0.08	148 ± 3
6b (6-ECDCA)	0.099 ± 0.01	144 ± 5
6c	1.11 ± 0.13	156 ± 2
6d	>30	5 ± 2

[a] Relative recruitment of the SRC1 peptide to FXR where CDCA (**1**) = 100% (ref 4). All data ± standard error, $n = 4$.

Scheme 1[a]



[a] Reagents and conditions: (1) pTsOH, 3,4-dihydropyrane, dioxane, 25 °C. (2) (a) LDA, R−Br (or Me−I for compound **9a**), THF, −78 °C; (b) 10% HCl, MeOH, reflux. (3) $NaBH_4$. (4) 10% NaOH, MeOH.

Results. Initially, we surveyed a number of compounds previously synthesized in our laboratory[12] and found that 6α-methyl-chenodeoxycholic acid (6-MeCDCA, **6a**) was a more potent FXR agonist than CDCA (**1**) (Table 1). This result prompted us to synthesize CDCA analogues **6b**−**d** characterized by increasingly bulkier 6α-substituents. The synthetic route to the 6α-alkyl CDCA analogues **6a**−**d** is outlined in Scheme 1. Treatment of the 3-tetrahydropyranyloxy derivative **8** of 7-keto-lithocholic acid (**7**) with methyl-, ethyl-, propyl-, and benzyl bromide, respectively, at −78 °C using lithium diisopropylamide as a base and HMPTA/tetrahydrofuran (THF) as solvents, followed by treatment with methanolic HCl, afforded the corresponding methyl 3α-hydroxy-7keto-6α-methyl-5β-cholan-24-oate (**9a**), methyl 3α-hydroxy-7keto-6α-ethyl-5β-cholan-24-oate (**9b**), methyl 3α-hydroxy-7keto-6α-propyl-5β-cholan-24-oate (**9c**), and methyl 3α-hydroxy-7keto-6α-benzyl-5β-cholan-24-oate (**9d**) in 22, 12, 5, and 13% yield, respectively. Finally, selective reduction of the 6α-alkyl-7-keto bile acid methyl esters **9a**−**d** with sodium borohydride[13] and subsequent hydrolysis of the methyl ester with alkali afforded the desired 3α,7α-dihydroxy-6α-alkyl-5β-cholan-24-oic acids **6a**−**d**.[14] It is worth noting that in each case only the 6α-alkyl derivative is formed, a selectivity that

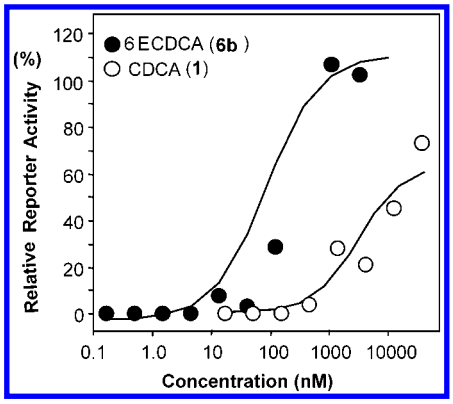


Figure 2. FXR Activity of **6b** in HuH7 cells. Data points represent the mean of assays performed in triplicate.

can be explained by the 1,3-diaxial interaction that would be experienced by a 6β-alkyl derivative with the C19 methyl group.

Biology: In Vitro Characterization. All new compounds were tested in an established cell-free ligand sensing assay, which measured the ligand-dependent recruitment of an SRC1 peptide to FXR by fluorescence resonance energy transfer.[4] The results, reported in Table 1, show that 6-ECDCA (**6b**) is a very potent FXR agonist with an EC_{50} of 99 nM. Also, the 6α-MeCDCA (**6a**) and 6α-PrCDCA (**6c**) derivatives demonstrated good potency as FXR agonists, while the 6α-BnCDCA derivative (**6d**) was essentially inactive.

In a reporter gene, (hsp70EcRE)2-tk-LUC,[6a] assay employing the full length human FXR in HuH7 cells, 6-ECDCA (**6b**) was a potent full agonist with an EC_{50} of 85 nM (Figure 2). When tested across a standard panel (described in ref 6a) of nuclear receptor LBD-GAL4 chimeric receptors,[4] 1 μM **6b** activated only the FXR(LBD)-GAL4 chimera (data not shown). No significant activation of other receptors was seen at 1 μM. Thus, **6b** is a potent and selective steroidal FXR agonist.

Biology: In Vivo Characterization in an Animal Model of Cholestasis. The most potent derivative 6-ECDCA (**6b**) was selected for further characterization in an in vivo model of cholestasis. Male Wistar rats (225−300 g) were catheterized at the right jugular vein using PE-50 polyethylene tubing, and the abdomen was opened through a midline incision. The common bile duct was isolated and cannulated. Saline solution was infused via the external jugular vein at the same infusion rate used later for BA, until a steady state in the bile flow was reached (75 min). BA were then dissolved in saline solution with 2% bovine serum albumin, pH 7.4, and infused for 90 min followed by 60 min of saline infusion. During the treatment, bile samples were collected every 15 min and weighed in order to determine the bile flow. Two protocols were used. In the first protocol, rats were randomly assigned to receive one of the following BA: LCA (**2**), 6-ECDCA (**6b**), or CDCA (**1**) at 1.0, 1.5, or 3 μmol/kg/min. 6-ECDCA (**6b**) was also infused at a higher dose (7 μmol/kg/min). In the second protocol, cholestasis was induced by intravenous infusion of LCA (**2**). Three groups of animals were treated as follows: LCA (**2**) (3 μmol/kg/min) alone, LCA (**2**) plus 6-ECDCA (**6b**) (3 μmol/kg/min), or LCA (**2**) plus CDCA (**1**) (3 μmol/kg/min). The results are shown in Figure 3. Administration of LCA alone at

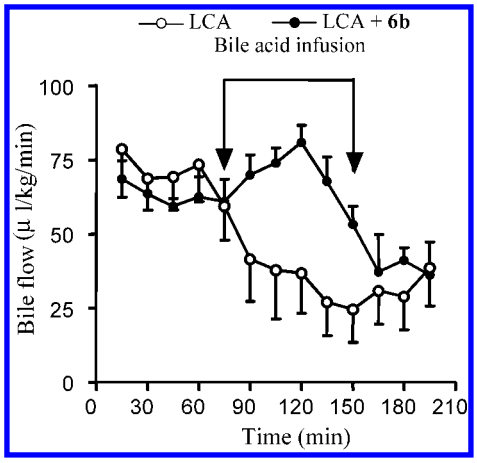


Figure 3. Effect of infusion of LCA alone (open circle) or in combination with **6b** (filled circle) on bile flow. Data are mean ± standard error of 4−6 rats/group.

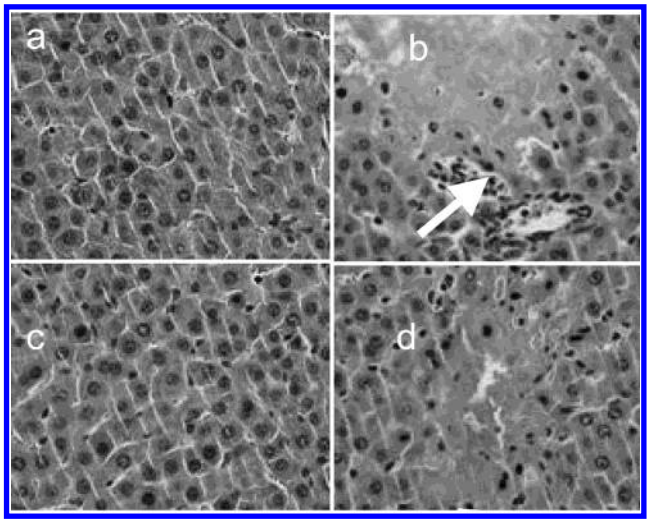


Figure 4. Histologic analysis of liver of rats treated with no agent (a), LCA alone (b), LCA plus **6b** (c), and LCA + CDCA (d). Each compound was infused at the concentration of 3 μmol/kg/min. Arrows indicate necrotic areas. Haematoxylin and Eosin staining, original magnification 200×.

a rate of 3 μmol/kg/min caused a dramatic reduction in bile flow and extensive necrosis of liver cells (Figure 4b, arrow).

At lower doses, LCA (**2**) was unable to induce cholestasis. Neither CDCA (**1**) nor 6-ECDCA (**6b**) induced cholestasis when administered alone. In the coinfusion protocol, 6-ECDCA (**6b**) dosed at a rate of 3 μmol/kg/min fully reversed the impairment of bile flow and protected against liver cell injury induced by 3 μmol/kg/min of LCA (**2**) (Figure 4c). Although 6-ECDCA (**6b**) was effective in protecting against cholestasis induced by LCA (**2**), the effect was lost over time, and a drop in bile flow was observed as expected when the infusion of the compound was stopped. In contrast, CDCA (**1**) at doses up to 7 μmol/kg/min failed to protect against colestasis caused by LCA (**2**) (data not shown), consistent with its low FXR affinity. Higher doses of CDCA (**1**) coinfused with LCA (**2**) were lethal (data not shown).

Discussion. The quest for potent and selective FXR ligands is driven by the need to further clarify the physiological and pathophysiological role of this nuclear BA receptor. We have reported here the synthesis and the preliminary evaluation of a series of potent steroidal

FXR agonists. Compound **6b** is almost 2 orders of magnitude more potent than CDCA (**1**), the putative physiological FXR ligand. Moreover, the synthesis of a series of 6α-substituted CDCA derivatives has allowed us to delineate a key structure−activity relationship within the steroidal skeleton. Indeed, our results point to the existence of a discrete hydrophobic pocket in the receptor, whose size is particularly suited to small linear alkyl substituents. Although further experiments are needed to fully characterize compounds **6a**−**d**, they appear to be useful chemical tools to probe the biological function of FXR. Thus, **6b** was active in an in vivo model of cholestasis, and when infused to rats codosed with LCA (**2**), it prevented bile flow impairment caused by this agent. Remarkably, despite its high lipophilicity, **6b** had no intrinsic cholestatic activity and did not show any evidence of acute liver toxicity.

In conclusion, we report the identification of a potent and selective steroidal FXR agonist, 6-ECDCA (**6b**). Importantly, not only did 6-ECDCA (**6b**) promote bile flow but it also protected hepatocytes against acute necrosis caused by LCA. Thus, FXR activation not only regulates endogenous bile acid synthesis but also activates protective pathways in hepatocytes challenged with toxic xenobiotics. 6-ECDCA (**6b**) and related analogues may, therefore, offer a rational approach to the treatment of cholestatic liver disease. Studies aimed at further elucidating the potential of FXR modulators in cholestasis as well as in other therapeutic areas are under way.

Acknowledgment. We thank Cristin Galardi and Kelli Plunket for determination of the activity of **6b** in the cell-based reporter assays.

References

(1) Mangelsdorf, D. J.; Evans, R. M. The RXR heterodimers and orphan receptors. *Cell* **1995**, *83*, 841−850.

(2) Forman, B. M.; Goode, E.; Chen, J.; Oro, A. E.; Bradley, D. J.; Perlmann, T.; Noonan, D. J.; Burka, L. T.; McMorris, T.; Lamph, W. W.; Evans, R. M.; Weinberger, C. W. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* **1995**, *81*, 687−693.

(3) (a) Makishima, M.; Okamoto, A. Y.; Repa, J. J.; Tu, H.; Learned, R. M.; Luk, A.; Hull, M. V.; Lustig, K. D.; Mangelsdorf, D. J.; Shanz, B. Identification of a nuclear receptor for bile acids. *Science* **1999**, *284*, 1362−1365. (b) Parks, D. J.; Blanchard, S. G.; Bledsoe, R. K.; Chandra, G.; Consler, T. G.; Kliewer, S. A.; Stimmel, J. B.; Willson, T. M.; Zavacki, A. M.; Moore, D. D.; Lehmann, J. M. Bile acids: natural ligands for an orphan nuclear receptor. *Science* **1999**, *284*, 1365−1368. (c) Wang, H.; Chen, J.; Hollister, K.; Sowers, L. C.; Forman, B. M. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell* **1999**, *3*, 543−553.

(4) Maloney, P. R.; Parks, D. J.; Haffner, C. D.; Fivush, A. M.; Chandra, G.; Plunket, K. D.; Creech, K. L.; Moore, L. B.; Wilson, J. G.; Lewis, M. C.; Jones, S. A.; Willson, T. M. Identification of a chemical tool for the orphan nuclear receptor FXR. *J. Med. Chem.* **2000**, *43*, 2971−2974.

(5) McKenna, N. J.; O'Malley, B. W. Combinatorial Control of Gene Expression by Nuclear Receptors and Coregulators. *Cell* **2002**, *108*, 465−474.

(6) (a) Goodwin, B.; Jones, S. A.; Price, R. R.; Watson, M. A.; McKee, D. D.; Moore, L. B.; Galardi, C.; Wilson, J. G.; Lewis, M. C.; Roth, M. E.; Maloney, P. R.; Willson, T. M.; Kliewer, S. A. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol. Cell* **2000**, *6*, 517−526. (b) Lu, T. T.; Makishima, M.; Repa, J. J.; Schoonjans, K.; Kerr, T. A.; Auwerx, J.; Mangelsdorf, D. J. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol. Cell* **2000**, *6*, 507−515.

(7) Willson, T. M.; Jones, S. A.; Moore, J. T.; Kliewer, S. A. Chemical genomics: functional analysis of orphan nuclear receptors in the regulation of bile acid metabolism. *Med. Res. Rev.* **2001**, *21*, 513−522.

(8) Chawla, A.; Repa, J. J.; Evans, R. M.; Mangelsdorf, D. J. Nuclear receptors and lipid physiology: opening the X-files. *Science* **2001**, *294*, 1866−1870.

(9) Sinal, C. J.; Tohkin, M.; Miyata, M.; Ward, J. M.; Lambert, G.; Gonzalez, F. J. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **2000**, *102*, 731−744.

(10) Hofmann, A. F. The continuing importance of bile acids in liver and intestinal disease. *Arch. Intern. Med.* **1999**, *159*, 2647−2658.

(11) Beuers, U.; Boyer, J. L.; Paumgartner, G. Ursodeoxycholic acid in cholestasis: potential mechanisms of action and therapeutic applications. *Hepatology* **1998**, *29*, 477−482.

(12) (a) Roda, A.; Pellicciari, R.; Cerre', C.; Polimeni, C.; Sadeghpour, B.; Marinozzi, M.; Cantelli Forti, G.; Sapigni, E. New 6-substituted bile acids: physicochemical and biological properties of 6α-methyl ursodeoxycholic acid and 6α-methyl-7-epicholic acid. *J. Lipid Res.* **1994**, *35*, 2268−2279. (b) Aldini, R.; Roda, A.; Montagnani, M.; Cerre', C.; Pellicciari, R.; Roda, E. Relationship between structure and intestinal absorption of bile acids with a steroid or side-chain modification. *Steroids* **1996**, *61*, 590−597.

(13) Mosback, E. H.; Meyer, W.; Kendall, F. E. Preparation and $NaBH_4$ reduction of 7-ketocholanic acid. *J. Am. Chem. Soc.* **1954**, *76*, 5799−5801.

(14) Selected analytical data for 6-EDCA (**6b**). ^{1}H NMR ($CDCl_3$): δ 0.67 (s, 3H, CH_3-18); 0.90−0.96 (m, 9H, CH_2-CH_3-6, CH_3-19, and CH_3-21); 2.22−2.46 (2m, 2H, CH_2-23); 3.39−3.47 (m, 1H, CH-3), 3.72 (brs, 1H, CH-7). ^{13}C NMR ($CDCl_3$): δ 11.65; 11.80; 18.25, 20.76; 22.23; 23.14; 23.69; 28.17; 30.53; 30.81; 30.95; 33.23; 33.90; 35.38; 35.52; 35.70; 39.60; 40.03; 41.19; 42.77; 45.19; 50.49; 55.80; 70.97; 72.38; 179.19. GC-MS methyl ester-trimethylsylyl ether derivative of 6-ECDCA, *m*/*z* (relative intensity): 579 ($M + H^+$, 1); 398 (base peak, 100).

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