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Identification of putative metabolites of docosahexaenoic acid as potent PPARγ agonists and antidiabetic agents

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Abstract—We found that putative metabolites of docosahexaenoic acid (DHA) are strong PPARγ activators and potential antidiabetic agents. We designed DHA derivatives based on the crystal structure of PPARγ, synthesized them and evaluated their activities in vitro and in vivo. The efficacy of 5*E*-4-hydroxy-DHA **2a** as a PPARγ activator was about fourfold stronger than that of pioglitazone. Furthermore, the 4-keto derivative (**10b**) showed antidiabetic activity in animal models without producing undesirable effects such as obesity and hepatotoxicity.

Peroxisome proliferator-activated receptors (PPARα, γ, δ) are nuclear receptors, which act as biosensors of fatty acids and play key roles in fatty acid metabolism and homeostasis. Disorders of lipid metabolism are a major threat to human health, leading to obesity, atherosclerosis, cardiovascular diseases, insulin resistance, and type 2 diabetes. The incidence of type 2 diabetes has increased dramatically over the past two decades, and it is now becoming one of the biggest public-health problems worldwide. It is well accepted that PPARγ is a target of antidiabetic agents such as thiazolidinediones (TZDs).[1,2] In agreement with this, mutations in the ligand-binding domain (LBD) of PPARγ in subjects exhibiting severe insulin resistance and early-onset type 2 diabetes mellitus have been reported.[3] Although TZDs are widely used, they have secondary effects such as obesity, edema and hepatotoxicity.[4,5] Recently, much attention has been focused on alternatives such as carboxylic acid derivatives, which are agonists of both PPARα and γ,[6,7] since the high PPARγ selectivity of TZDs is thought to be a cause of their side effects.[8,9]

Based on the idea that, for chronic diseases, compounds that are closely related to natural substances would be the drugs of better choice, we became interested in developing fatty acid derivatives as potent antidiabetic agents that would target PPARγ. Some arachidonic acid metabolites, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ2), are natural ligands with moderate affinity for PPARγ.[10] However, they have not been reported to have antidiabetic activity in vivo. We focused our attention on docosahexaenoic acid (DHA) derivatives, because DHA (**1**) has long been used as a functional food worldwide and is known to have beneficial effects in several human diseases including atherosclerosis, asthma, cardiovascular disease, cancer, and depression.[11] Recently, various oxidative metabolites of DHA have been isolated and reported to exert anti-inflammatory effects.[12] However, their potency in transactivation of PPARγ remains unknown. In this paper we report the design of various DHA derivatives that bind to PPARγ, based on the crystal structure of PPARγ/agonist complex. We found that several of these

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compounds show higher activity in transactivation of PPARγ than PGJ2 and that some have even higher activity than a TZD derivative, pioglitazone. These DHA derivatives are putative metabolites or their related compounds.[12–14] We further demonstrated that at least one of these derivatives has useful antidiabetic activity in vivo.

We designed DHA derivatives (Fig. 1) that might have higher activity than the parent compound based on the crystal structure of the PPARγ–LBD/rosiglitazone complex[15] and using docking software FLEXX (Tripos, St. Louis). The crystal structure indicated that rosiglitazone docks into the ligand-binding pocket (LBP) of PPARγ, with its TZD ring oriented towards the AF2 helix and forming hydrogen bonds with five residues, Q286 (H3), S289 (H3), H323 (H4), H449 (H11), and Y473 (H12) (Fig. 2a). TZD derivatives are poor PPARα agonists, because the TZD head group creates a steric clash with Y314, which corresponds to H323 in PPARγ.[6,7] Our docking analysis indicated that parent DHA (**1**) is easily accommodated in the PPARγ–LBP, and that the carboxyl group forms hydrogen bonds with four of the five residues that form hydrogen bonds with the TZD ring (S289, H323, H449, and Y473) (Fig. 2b). In order to design DHA derivatives as potent PPARγ agonists, we searched for hydrophilic residues that would face DHA when it is docked into the LBP and would be able to form the fifth hydrogen bond. We paid attention to Y327 in helix 4/5 whose hydroxyl group faces the C(4) of DHA. Thus, we designed a key compound, 5*E*-4-hydroxydocosahexaenoic acid (4-OHDHA, **2a**), which has a hydroxyl group at C(4). As shown in Figure 2c, the 4-hydroxyl group of (4*S*)-**2a** does indeed form a hydrogen bond with Y327. Lactones, **3** and **6**, are precursors of 4-OHDHA (**2a**) in the synthesis from DHA, and **6** may serve as a pro-drug of **2a**. Lactol **7** and diol **8** were also designed as possible pro-drugs of **2a**, since these are expected to be converted to **2a** by oxidation in vivo. The other compounds (**4**, **5**, and **9**–**13**) were designed to allow structure–activity relationship studies.

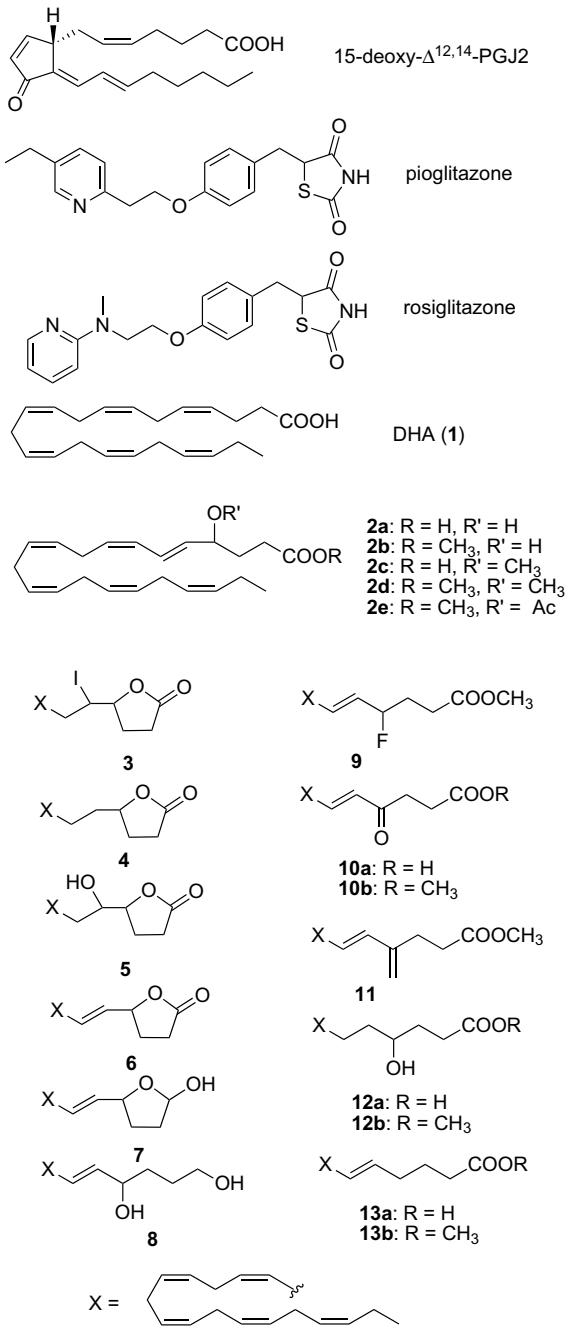


Figure 1. Structures of DHA derivatives.

A total of 19 DHA derivatives were synthesized as shown in Scheme 1. We used iodolactonization of DHA as the key reaction to introduce functional group at C(4). Thus, all the compounds except for the 5*E*-Δ^5-DHA derivatives (**13a** and **13b**) were synthesized from DHA via the key compound **3**. Compound **13a** and **13b** were synthesized by coupling of Wittig reagent **14** derived from EPA with aldehyde **15**. Compounds with asymmetric carbon at C(4) (**2**, **4**, **6**, **8**, **9**, and **12**) are racemic mixtures and those with two asymmetric carbons (**3**, **5**, and **7**) are a mixtures of possible four isomers. Structures of newly synthesized compounds were confirmed by spectral analyses.[16] Details of the synthetic procedures are described in a separate paper in preparation.

The transcriptional activities of the 19 DHA derivatives were evaluated in Cos7 cells by dual luciferase assays using a reporter plasmid containing four copies of MH100 GAL4 binding site (MH100 × 4-TK-Luc),[17] GAL4-hPPARγ chimera expression plasmid (pSG5-GAL-hPPARγ)[1] and an internal control plasmid containing sea pansy luciferase expression constructs (pRL-CMV).[18] The relative activities of the DHA derivatives compared with DHA, PGJ2, and pioglitazone are summarized in Table 1. All compounds tested had higher potency than the parent fatty acid (**1**) and several (**2a**, **9**, **10a**, and **10b**) showed activity comparable to or higher than that of pioglitazone. It should be noted that the compound with the highest activity, 4-OHDHA (**2a**), is a putative metabolite of DHA; DHA has been reported to yield some 4-hydroxylated metabolites in vitro under the influence of 5-lipoxygenase.[12] 4-Oxo-DHA **10a** may also be a metabolite produced by dehydrogenation of **2a**, since oxidation of 5-HETE to 5-oxo-ETE has been reported under similar conditions.[19]

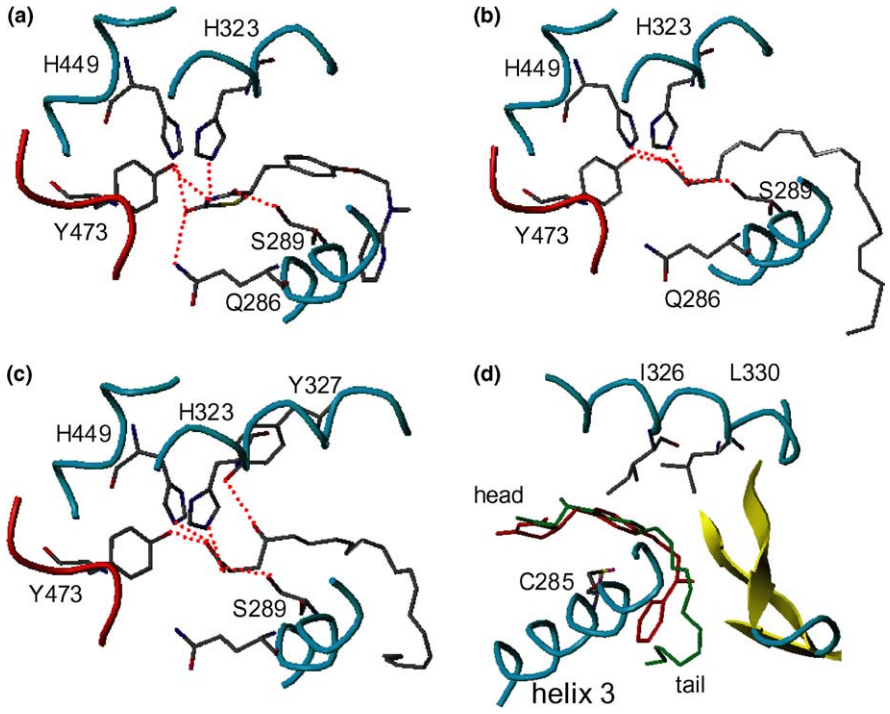
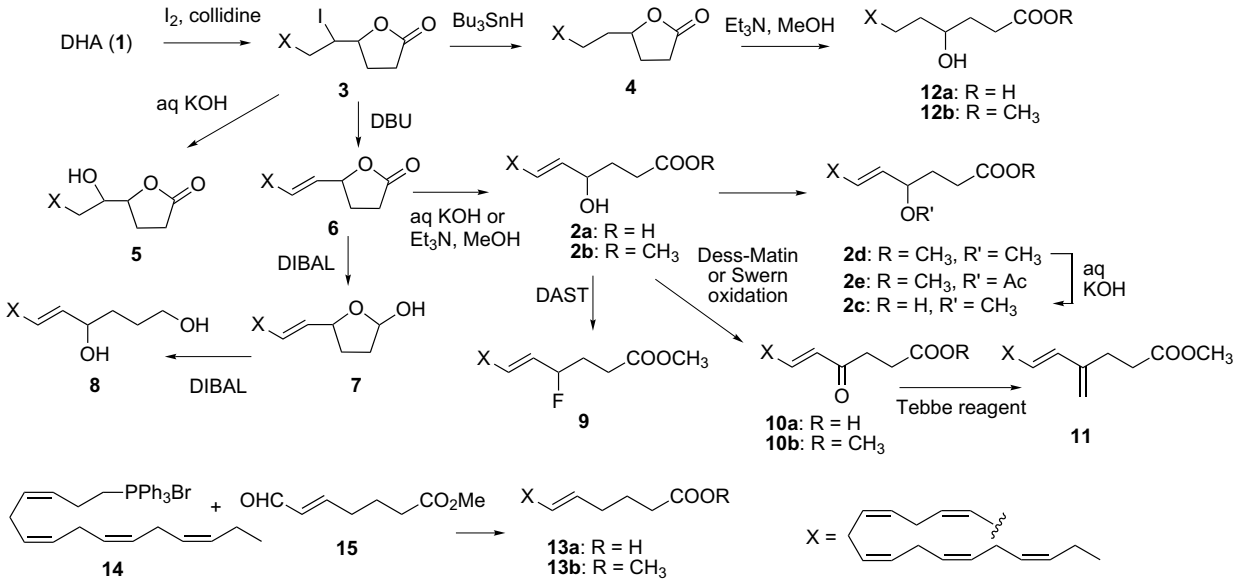


Figure 2. Structures of PPARγ–ligand complexes. Hydrogen bonds are depicted by red dotted lines: (a) crystal structure of the PPARγ–LBD and rosiglitazone complex.[13] The TZD ring forms hydrogen bonds with five residues, Q286, S289, H323, H449, and Y473; (b) model of the PPARγ and DHA (**1**) complex. The carboxyl group forms hydrogen bonds with only four residues, S289, H323, H449, and Y473; (c) model of the PPARγ and (4*S*)-4-OHDHA **2a** complex. A fifth hydrogen bond can be formed between the 4-hydroxyl group and Y327; (d) superimposition of (4*S*)-4-OHDHA **2a** and rosiglitazone in the PPARγ–LBP. The two ligands occupy almost the same positions in the pocket.



Scheme 1.

The dose–response results for the potent compounds **2a**, **10a**, and **10b** are interesting (Fig. 3a). Compounds **2a** and **10a** showed different patterns from both PGJ2 and pioglitazone. 4-OHDHA **2a** exhibited weaker activity than pioglitazone at 1 μM, but was stronger at 5 μM. It is known that a large proportion (>99%) of the free fatty acids in plasma is bound to serum albumin. Since the assay solution contained 5% FBS, we suppose that 4-OHDHA **2a** might bind to albumin and be unable to enter cells readily at low concentration. Therefore, we re-evaluated the activity of **2a** in the absence of FBS, and found that it showed higher activity than

Table 1. Activity of DHA derivatives on human PPARγ[a]

Compd	Activity[b]
Vehicle	1.0
PGJ2	5.9
Pioglitazone	6.9
DHA **1**	1.2
2a	13.4
2b	3.8
2c	1.7
2d	2.3
2e	1.9
3	1.4
4	3.5
5	1.4
6	2.5
7	5.7
8	5.1
9	6.0
10a	7.8
10b	6.6
11	1.5
12a	2.0
12b	1.7
13a	1.2
13b	1.3

[a] All compounds were tested at 5 μM in the presence of 5% FBS using Cos7 cells by dual luciferase assay.
[b] Activities are presented by fold induction of PPARγ activation.

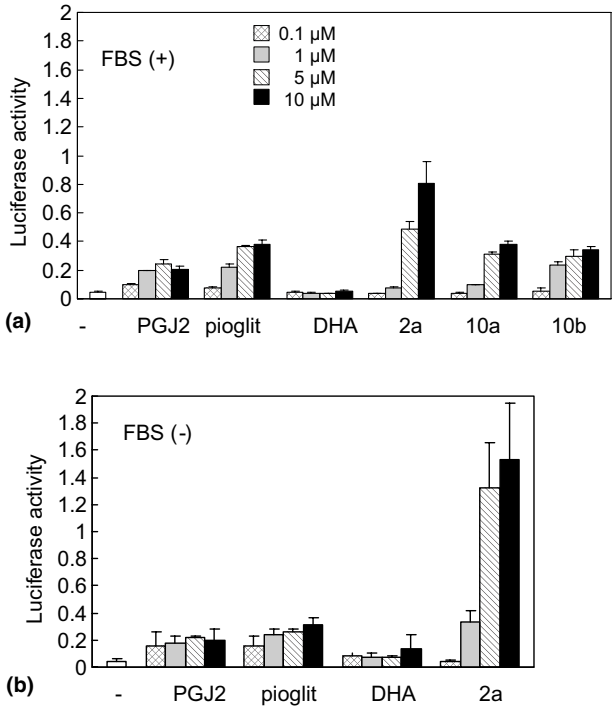


Figure 3. Dose–response relationship of the most potent compounds (**2a**, **10a**, and **10b**) with respect to PPARγ activation. Compounds were tested in the presence (a) or absence (b) of 5% FBS using Cos7 cells by dual luciferase assay: (a) in the presence of 5% FBS, **2a** and **10a** showed different dose–response patterns from both PGJ2 and pioglitazone. Compound **2a** showed weaker activity than pioglitazone at 1 μM but stronger activity at 5 μM; (b) in the absence of FBS, **2a** showed higher activity than pioglitazone even at 1 μM and the efficacy of **2a** was four-times stronger than that of pioglitazone.

pioglitazone even at 1 μM. Indeed, **2a** was more than four times as effective as pioglitazone (Fig. 3b). These results indicate that, once they have been transported into cells, at least some of the DHA derivatives have much higher activities than those observed in the presence of 5% FBS (Table 1).

Methyl ether **2c** and acetate **2e** showed significantly lower activity than **2a**, indicating the importance of the 4-hydroxyl group. As shown in Table 1, this hydroxyl group is replaceable with fluorine (**9**) or keto group (**10a** and **10b**), but not with hydrogen (**13a**) or methylene (**11**). The results emphasize the importance of a hydrophilic substituent at C(4). It should also be noted that saturation of the double bond at C(5) with hydrogen significantly lowered the potency, as the poor activity of compound **12** shows. This may be explained by the entropic effect of the rigid 5,7-conjugated diene system.[20] Thus, it is clear that both a hydrophilic group at C(4) and a 5*E*,7*Z*-conjugated diene structure are important in producing highly potent DHA derivatives. Lactol **7** and diol **8** showed significant activity, but it is not known whether these two compounds exhibit their activity after conversion to oxidative metabolites such as **2a**.

The in vivo antidiabetic activities of 4-OHDHA (**2a**) and keto ester **10b** were evaluated using male db/db mice and Zucker diabetic fatty (ZDF) rats. The db/db mouse bears a defect in the leptin receptor gene, which leads to an uncontrolled appetite to develop obesity-induced diabetes. ZDF rats are also deficient in leptin receptors. 4-OHDHA (**2a**) had little effect in db/db mice (data not shown), probably because of poor availability to the target tissues. Preliminary experiments in normal rats showed that absorption of **2a** into the lymph and serum is poor. On the other hand, as seen in Table 2, **10b** significantly reduced blood glucose (BG) levels in db/db mice at a dose of 10 mg/kg. The antidiabetic effect of **10b** was slightly lower than that of pioglitazone. Interestingly, no dose-dependent effect on the BG levels was apparent at a higher dose (100 mg/kg). In contrast to pioglitazone, **10b** had little effect on serum triglyceride (TG) levels. A similar activity profile was observed in the ZDF rats, in which **10b** also reduced BG but not TG levels. Thus, keto ester **10b** has an activity profile distinct from that of pioglitazone. Furthermore, in preliminary high-dose toxicity tests in normal rats (1, 0.2, and 0.04 g/kg for 14 days), **10b** produced neither obesity nor liver toxicity (data not shown). The distinct activity profile of **10b** in vivo may mean that it shows antidiabetic activity without producing the undesirable effect of obesity.

In the docking analysis, all of the potent compounds (**2a**, **9**, **10a**,**b**) were accommodated appropriately in the PPARγ–LBP and hydrogen-bonded with Y327. Figure 2d shows a superimposition of (4*S*)-**2a** and rosiglitazone in the PPARγ-LBP. Compound (4*S*)-**2a** adopts the same overall shape as rosiglitazone in the LBP. The tail of (4*S*)-**2a** is extended a little more toward the open channel between helix 3 and the β-sheet in comparison with rosiglitazone. The hydrogen bond between Y327

Table 2. In vivo activity of **10b** in animal models

Compound	Dose (mg/kg)	Blood glucose (mg/dL)	Triglyceride (mg/dL)	Body weight gain (g)
db/db Mouse[a]				
Vehicle	—	945 ± 122	193 ± 81	5.4 ± 0.6
Pioglitazone	100	432 ± 74**	73 ± 26**	15.5 ± 3.0**
10b	100	653 ± 66**	188 ± 66	3.2 ± 1.3
10b	10	514 ± 70**	138 ± 66	3.7 ± 0.9
ZDF rat[b]				
Vehicle	—	364 ± 80	819 ± 204	56.2 ± 16.3
Pioglitazone	30	174 ± 70**	109 ± 67**	96.6 ± 9.5**
10b	30	228 ± 49**	765 ± 157	61.9 ± 8.6
10b	3	262 ± 72*	958 ± 309	60.1 ± 9.9

[a] Six db/db mice/group at 8 weeks old were orally administered once daily with vehicle or **10b** in vehicle. After 4 weeks of dosing, mice were anesthetized with pentobarbital and blood was collected.

[b] Six ZDF rats/group at 9 weeks old were orally administered once daily with vehicle or **10b** in vehicle. After 2 weeks of dosing, rats were anesthetized with pentobarbital and blood was collected.

*$P < 0.05$.

**$P < 0.01$.

and 4-hydroxyl group must be essential because only compounds with a hydroxyl, carbonyl or fluorine group at C(4) activate PPARγ. As noted above, the planar 5*E*,7*Z*-conjugated diene structure is also important for high biological potency. This diene moiety fits into a narrow, hydrophobic tunnel formed by C285 (H3), L330 (H5), and I326 (H5), and overlaps with the middle aromatic ring of rosiglitazone, as seen in Figure 2d.

PPARγ plays an important role in energy accumulation, whereas PPARα is involved in energy consumption. Therefore, dual agonists of PPARγ and α are now thought to be good antidiabetic agents that will not give rise to undesirable effects such as obesity and edema.[21] We identified putative metabolites of DHA that strongly activate PPARγ, of which 4-keto ester **10b** showed antidiabetic activity in vivo without producing any side effects. These DHA derivatives may function as dual agonists, as they possess a carboxyl group but not a TZD ring.[6,7] Thus, we present here novel potential leads for treating type 2 diabetes. Although they are not as active as pure synthetic aromatic agents in vivo at the present time, they may be nontoxic agents that would not cause obesity during long-term treatment. Further studies are now needed to prove the potential of these compounds and to clarify their behavior in vivo.

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16. Spectral data. (5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-4-Hydroxy-5,7,10,13,16,19-docosahexaenoic acid (**2a**): ^{1}H NMR: δ 0.97 (3H, t, J = 7.5 Hz, H-22), 1.88 (2H, m, H-3), 2.08 (2H, m, H-21), 2.48 (2H, t, J = 7.3 Hz, H-2), 2.80–2.91 (6H, m, H-12, 15, 18), 2.97 (2H, t, J = 6.6 Hz, H-9), 4.25 (1H, dd, J = 12.6, 6.6 Hz, H-4), 5.32–5.43 (9H, m, H-8, 10, 11, 13, 14, 16, 17, 19, 20), 5.68 (1H, dd, J = 15.1, 6.5 Hz, H-5), 6.01 (1H, t, J = 11.0 Hz, H-7), 6.54 (1H, dd, J = 15.1, 11.0 Hz, H-6); MS *m*/*z* 344 (M^+, 1), 327 (3), 326 (7), 297 (4), 246 (14), 187 (16), 117 (50), 108 (55), 79 (100); HRMS Calcd for $C_{22}H_{30}O_2$ (M^+-H_2O) 326.2246. Found 326.2256. Methyl(5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-4-oxo-5,7,10,13,16,19-docosahexaenoate (**10b**): ^{1}H NMR: δ 0.97 (3H, t, J = 7.5 Hz, H-22), 2.07 (2H, m, H-21), 2.66 (2H, t, J = 6.7 Hz, H-2), 2.77–2.88 (6H, m, H-12, 15, 18), 2.92 (2H, t, J = 6.7 Hz, H-3), 3.01 (2H, t, J = 7.3 Hz, H-9), 3.69 (3H, s, CO_2Me), 5.28–5.49 (8H, m, H-10, 11, 13, 14, 16, 17, 19, 20), 5.87 (1H, m, H-6), 6.15 (1H, d, J = 11.4 Hz,

H-8), 6.20 (1H, d, $J = 15.5$ Hz, H-5), 7.56 (1H, dd, $J = 15.5$, 11.4 Hz, H-7); ^{13}C NMR: δ 14.3, 20.6, 25.6, 25.7, 25.8, 26.7, 28.0, 35.5, 51.9, 126.4, 127.0, 127.1, 127.7, 128.6, 128.7, 129.5, 129.7, 132.1, 137.1, 140.1, 173.4, 198.3; MS *m*/*z* 356 (M^+, 5), 276 (7), 189 (22), 167 (25), 137 (25), 115 (100), 79 (43); HRMS Calcd for $C_{23}H_{32}O_3$ (M^+) 356.2351. Found 356.2335.

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