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The Amide Hydrogen of (−)-Indolactam-V and Benzolactam-V8's Plays a Critical Role in Protein Kinase C Binding and Tumor-Promoting Activities

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Abstract—To investigate the role of the amide hydrogen of (−)-indolactam-V (**1**) and benzolactam-V8's on protein kinase C (PKC) binding and tumor promotion, 8-decylbenzolactone-V8 (**6**), a new lactone analogue of 8-decylbenzolactam-V8 (**4**), was synthesized from 2-nitrophenylpyruvic acid (**7**) in 11 steps. The PKC binding ability and tumor-promoting activities in vitro of **6** were much lower than those of **1** and **4**, suggesting that the amide hydrogen of **1** and benzolactam-V8's plays a critical role in tumor promotion. However, it is noteworthy that **6** showed significant selectivity in the PKC isozyme surrogate binding. © 2001 Published by Elsevier Science Ltd.

Tumor-promoting (−)-indolactam-V (**1**)[1,2] is the minimal basic structure exhibiting tumor promotion and activation of protein kinase C (PKC),[3] a crucial enzyme involved in cellular signal transduction (Fig. 1). Intensive structure–activity studies on **1** revealed almost all structural factors required for tumor promotion and PKC binding except for the role of the amide subunit.[4] Conformation studies led to the finding that **1** exists as two stable conformers in solution at room temperature;[5] the active twist conformer with a *cis* amide geometry and the inactive sofa conformer with a *trans* amide geometry.[6,7] The tumor promoter binding site of PKC was also identified; phorbol ester type tumor promoters bind to the cysteine-rich C1 domains designated as C1A and C1B.[8,9] Based on these findings coupled with X-ray crystal structure analysis of PKCδ-C1B in complex with phorbol 13-acetate as a ligand,[10] computational docking studies indicated that both the amide hydrogen and the carbonyl oxygen of **1** interact with the binding site of PKCδ-C1B.[11,12] However, there are no experimental results on the role of the amide group in the PKC isozyme binding and tumor promotion. Although we previously synthesized indolactone-V (**2**), a lactone analogue of **1**, we could not determine whether the amide hydrogen of **1** is necessary for tumor promotion or not since **2** existed as only the inactive sofa conformer.[13]

Endo et al. and Kozikowski et al. independently reported that benzolactam-V8 (**3**) with an eight-membered lactam containing a benzene ring instead of the nine-membered lactam of **1** reproduced the active conformation of **1**. 8-Decylbenzolactam-V8 (**4**) showed significant tumor-promoting activities in vitro comparable to **1**.[6,14,15] They also independently found that the binding mode of **3** to PKCδ-C1B is quite similar to that of **1** by computational docking studies.[11,15] These findings prompted us to synthesize 8-decylbenzolactone-V8 (**6**), a new lactone analogue of **4**, and to examine its conformation, PKC binding ability, and tumor-promoting activities in vitro.

Benzolactone-V8 (**5**), the core structure of 8-decylbenzolactone-V8 (**6**), was synthesized from 2-nitrophenylpyruvic acid (**7**) as shown in Scheme 1. Reduction of both the ketone and the carboxyl group of **7** with

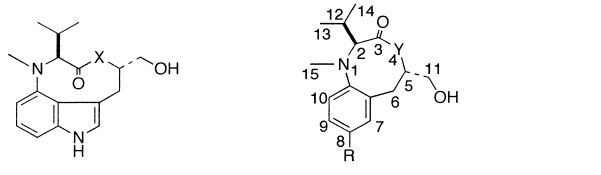

Figure 1. Structures of (−)-indolactam-V, benzolactam-V8's, and their lactone derivatives.

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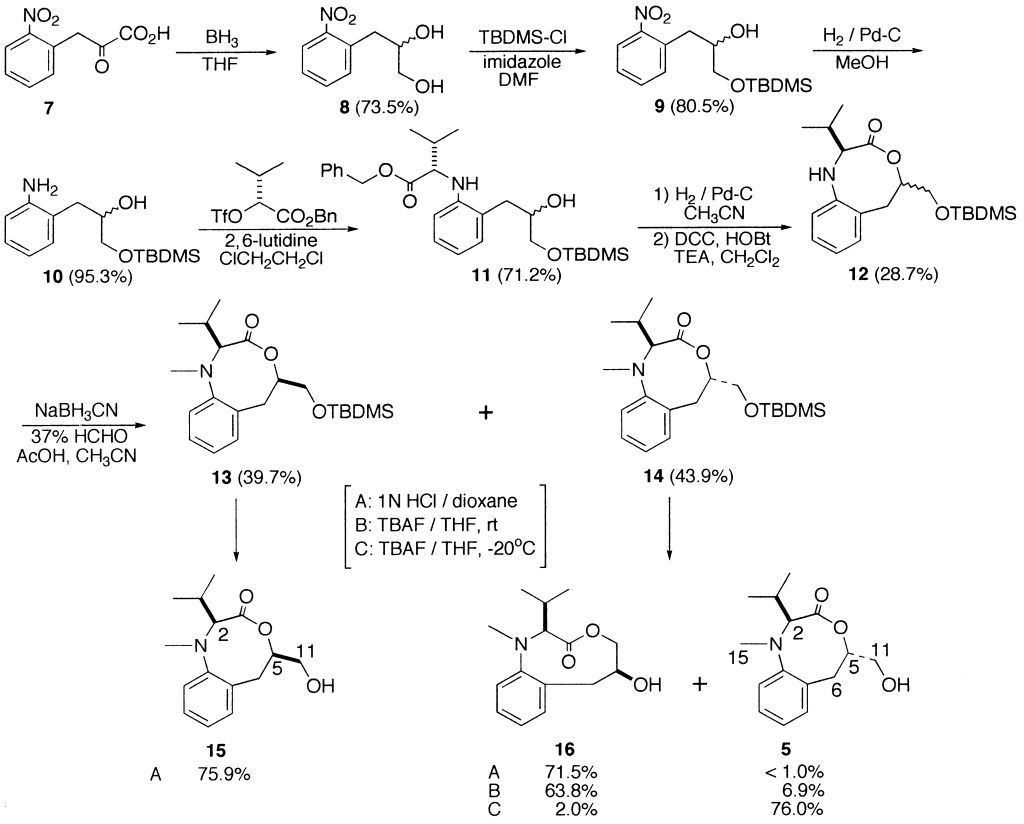


Scheme 1. Synthesis of benzolactone-V8 (**5**).

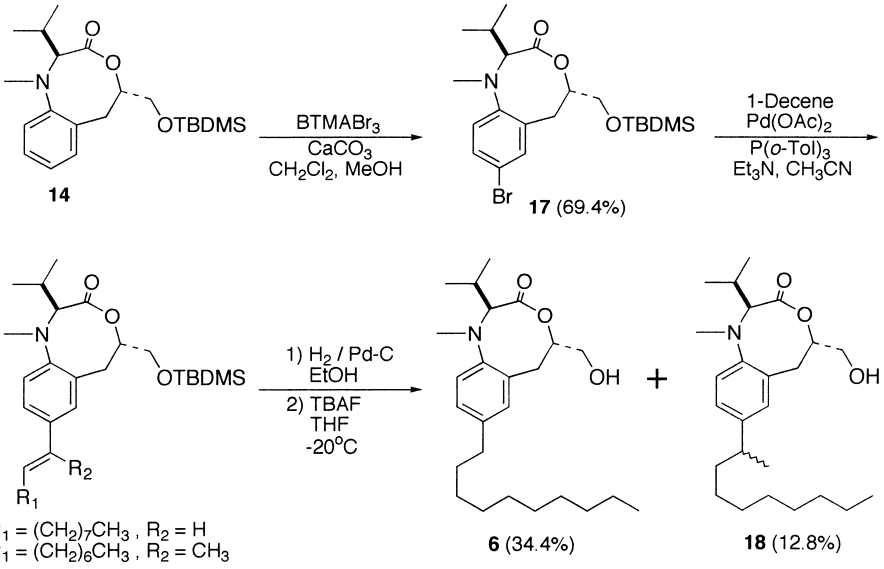
borane gave a diol (**8**, 73.5%), whose primary hydroxyl group was protected selectively with a *tert*-butyldimethylsilyl (TBDMS) group (80.5%). The nitro group of **9** was reduced by catalytic hydrogenation using 10% palladium on carbon to give the aniline derivative **10** (95.3%). The valine subunit was introduced by substitution of **10** with D-valine-derived triflate[16] to give two diastereomeric esters (**11**, 71.2%). Since separation of these diastereomers was quite difficult at this point, we proceeded to the next cyclization step. After deprotection of the benzyl group of **11** by hydrogenation in acetonitrile, intramolecular esterification was accomplished with DCC, HOBt, and triethylamine in dichloromethane to give **12** (28.7%). This slightly lower cyclization yield was expected since the synthesis of an eight- or a nine-membered lactone is generally quite difficult.[17] The presence of bulky substituents like isopropyl and TBDMS groups might be another reason for this low yield. Methylation of the diastereomeric lactones (**12**) by the method of Kozikowski et al.[15] gave two diastereomers (**13**, **14**), which were easily separated by silica gel column chromatography.

Deprotection of each TBDMS group of **13** and **14** with 1 N HCl in dioxane gave a single product, **15** and **16**, respectively, whose ^{1}H NMR spectra in deuteriochloroform showed that each compound existed as a single conformer at room temperature (Table 1). The ^{1}H–^{1}H COSY spectrum indicated that **15** was an eight-membered lactone since the cross peak between the hydroxyl group and methylene protons at position 11 was observed. The significant NOE between H-2 and H-5 protons in the NOESY spectrum suggested that **15** was *epi*-benzolactone-V8[18] with the *R* configuration at position **5**. On the other hand, the ^{1}H–^{1}H COSY spectrum revealed that **16** was not the expected benzolactone-V8 but the nine-membered lactone[19] because of the lack of the hydroxymethyl proton signals. This nine-membered lactone (**16**) was deduced to be formed by the intramolecular transesterification under the acidic condition of the TBDMS deprotection step (1 N HCl–dioxane). We attempted to deprotect the TBDMS group of **14** with TBAF in THF at room temperature to get a major product identified as **16** (63.8%) along with a minor product **5** (6.9%), which was thought to be a desired eight-membered lactone. To suppress the transesterification, we conducted this reaction at −20 °C and succeeded in obtaining mainly **5** (76.0%). The ^{1}H NMR spectrum showed that **5** existed as a single conformer in deuteriochloroform at room temperature. The cross peak between the hydroxyl group and the methylene protons at position 11 was observed in the ^{1}H–^{1}H COSY spectrum, suggesting that **5** is an eight-membered lactone. No NOE enhancement between H-2 and H-5 protons in the NOESY spectrum strongly indicated that **5** is benzolactone-V8[20] with the *S* configuration at position 5. In addition, significant NOE enhancements between H-2 and H-6 protons, and between H-5 and H-15 protons, which are characteristic of benzolactam-V8 (**3**),[6] were observed, indicating that the conformation of **5** is very close to that of **3**. Benzolactone-V8 (**5**) did not convert to **16** even at room temperature in Tris or phosphate buffer (pH 7.4) which is used in the bioassays shown below.

Table 1. ^{1}H NMR spectra of benzolactam-V8 (**3**), benzolactone-V8 (**5**), *epi*-benzolactam-V8, and *epi*-benzolactone-V8 (**15**) in deuteriochloroform (500 MHz, 300 K)

No	δ (Multiplicity, *J* in Hz)			
	Benzolactam-V8 (**3**)[a]	Benzolactone-V8 (**5**)[b]	*epi*-Benzolactam-V8[c]	*epi*-Benzolactone-V8 (**15**)[d]
2	3.46 (1H, d, *J* = 8.6)	3.34 (1H, d, *J* = 10.2)	3.18 (1H, d, *J* = 10.6)	3.28 (1H, d, *J* = 10.8)
5	4.05 (1H, m)	4.82 (1H, m)	3.83 (1H, m)	4.65 (1H, m)
6a	2.81 (1H, dd, *J* = 16.9, 2.2)	2.98 (1H, dd, *J* = 16.3, 5.1)	2.86 (1H, d, *J* = 15.2)	2.82 (1H, dd, *J* = 15.5, 2.4)
6b	3.08 (1H, dd, *J* = 16.9, 8.0)	3.05 (1H, dd, *J* = 16.3, 3.7)	2.92 (1H, dd, *J* = 15.2, 6.5)	2.94 (1H, dd, *J* = 15.5, 5.8)
7	7.02 (1H, d, *J* = 7.6)	7.08 (1H, d, *J* = 7.6)	7.10 (1H, d, *J* = 7.4)	7.11 (1H, d, *J* = 7.3)
8	7.18 (1H, t, *J* = 7.6)	7.22 (1H, t, *J* = 7.6)	7.19 (1H, t, *J* = 7.4)	7.22 (1H, t, *J* = 7.3)
9	6.88 (1H, t, *J* = 7.6)	7.04 (1H, t, *J* = 7.6)	6.95 (1H, t, *J* = 7.4)	7.06 (1H, t, *J* = 7.3)
10	7.04 (1H, d, *J* = 7.6)	7.09 (1H, d, *J* = 7.6)	7.12 (1H, d, *J* = 7.4)	7.19 (1H, d, *J* = 7.3)
11a	3.52 (1H, m)	3.69 (1H, m)	3.76 (1H, m)	3.77 (1H, dd, *J* = 11.9, 4.2)
11b	3.70 (1H, m)	3.69 (1H, m)	3.76 (1H, m)	3.82 (1H, dd, *J* = 11.9, 7.2)
12	2.43 (1H, m)	2.23 (1H, m)	2.41 (1H, m)	2.32 (1H, m)
13	1.06 (3H, d, *J* = 6.5)	1.02 (3H, d, *J* = 6.6)	0.97 (3H, d, *J* = 6.6)	1.06 (3H, d, *J* = 6.6)
14	0.89 (3H, d, *J* = 6.8)	0.99 (3H, d, *J* = 6.5)	0.87 (3H, d, *J* = 6.5)	0.88 (3H, d, *J* = 6.5)
15	2.79 (3H, s)	2.80 (3H, s)	2.93 (3H, s)	2.91 (3H, s)

[a]0.082 M.
[b]0.102 M.
[c]0.067 M.
[d]0.061 M.



Scheme 2. Synthesis of 8-decylbenzolactone-V8 (**6**).

A decyl group was introduced at position 8 of **14** as shown in Scheme 2. Unexpectedly, iodination of **14** using iodine in pyridine did not proceed at all though 11-*O*-acetylbenzolactam-V8 was iodinated by the same reaction conditions.[15] Since transesterification might occur in the conventional iodination and bromination reactions under strong acidic conditions, we used a strong bromination reagent, benzyltrimethylammonium tribromide ($BTMABr_3$),[21] under neutral conditions. Bromination of **14** using $BTMABr_3$ mildly proceeded at room temperature to give 11-*O*-TBDMS-8-bromobenzolactone-V8 (**17**, 69.4%). Coupling reaction of **17** with 1-decene was accomplished by the method of Endo et al.[6] to give two coupling products. Hydrogenation of these alkenes using 5% palladium on carbon followed by deprotection of the TBDMS group with TBAF at −20 °C gave 8-decylbenzolactone-V8 (**6**, 34.4%)[22] and its regio isomers (**18**, 12.8%). The ^{1}H NMR and NOESY spectra showed that **6** existed as a single conformer in deuteriochloroform at room temperature, and that its ring conformation is quite similar to that of benzolactam-V8 (**3**).

The biological activities of **6** along with **1** and **4**, which was synthesized by the method of Endo et al.[14] with a slight modification, were examined by three in vitro bioassays related to in vivo tumor promotion: binding to the PKC C1 domains,[23,24] Epstein-Barr virus early antigen (EBV-EA)-inducing ability,[25,26] and superoxide (O_2^-) generation-inducing ability in differentiated HL-60 cells.[27,28] The binding affinity to the PKC C1 domains was evaluated by inhibition of the specific binding of [^{3}H]phorbol-12,13-dibutyrate (PDBu) to these C1 domains as reported by Sharkey and Blum-

berg.[24] We have recently synthesized individual C1A and C1B domains of all PKC isozymes consisting of about 50 amino acids by the solid-phase synthesis and measured the dissociation constants (K_d) of [^{3}H]PDBu.[23,29,30] Using these PKC C1 peptides, the concentration required to cause 50% inhibition, IC_{50}, of the [^{3}H]PDBu binding was measured. The binding affinity of **4** and **6** to each PKC C1 peptide was expressed as the K_i values calculated from the IC_{50} and the K_d values of [^{3}H]PDBu as reported previously.[24,29,30]

EBVs are under the strict control of the host human lymphoblastoid Raji cells. They are activated by tumor promoters to produce the early antigen (EA).[25,26] The EBV-EA-inducing activity is expressed as the percentage of EA-positive cells. Under our experimental conditions, about 30% of maximum EA-induction was observed with typical tumor promoters.[7] Superoxide (O_2^-) generation is triggered by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in epithelial cells and leukocytes through the xanthine oxidase[31] and NADPH oxidase systems,[32] respectively. The ability is expressed as the level of O_2^- production. Under our experimental conditions, TPA produced 1.32 nmol/mL/min of O_2^- at 10^{-7} M. Tables 2–4 summarize the results of these assays.

8-Decylbenzolactam-V8 (**4**) showed slightly weaker binding affinities than **1** for almost all PKC C1 peptides. On the other hand, the binding affinities of 8-decylbenzolactone-V8 (**6**) for all PKC C1 peptides were far lower than those of **1** and **4**. These results indicate that the amide hydrogen of **1** and benzolactam-V8's is necessary to amplify the binding affinities for all PKC isozymes.

Table 2. K_i Values for inhibition of the specific binding of [^{3}H]PDBu by 8-decylbenzolactam-V8 (**4**) and 8-decylbenzolactone-V8 (**6**)

PKC C1 peptide	K_i (nM)		
	8-Decylbenzolactam-V8 (**4**)	8-Decylbenzolactone-V8 (**6**)	(−)-Indolactam-V (**1**)[a]
α-C1A(72-mer)[b]	322.5 (36.1)[c]	>10,000	126.9
α-C1B	4690 (201)	>10,000	4000
β-C1A(72-mer)[b]	442.4 (34.7)	>10,000	173.5
β-C1B	260.8 (16.7)	22,010 (1896)	135.6
γ-C1A	1664 (99.7)	6321 (536)	137.9
γ-C1B	150.6 (6.8)	17,574 (1648)	212.6
δ-C1A	2771 (240)	70,641 (3322)	1900
δ-C1B	14.6 (1.6)	1155 (46)	8.3
ε-C1A	8361 (385)	>10,000	4110
ε-C1B	13.1 (0.2)	262.3 (3.5)	7.7
η-C1A	2489 (74)	>10,000	3770
η-C1B	6.4 (1.2)	119.9 (6.7)	5.5
θ-C1A	NT[d]	NT	NT
θ-C1B	13.2 (2.6)	1097 (53)	8.7

[a]Data taken from ref 30.
[b]Ten residues from both N- and C-termini of the previous α-C1A and β-C1A[23] were elongated since the solubility of the original 52-mer peptides was extremely low.
[c]Standard deviation of at least two separate experiments.
[d]Not tested. The K_d value of [^{3}H]PDBu to θ-C1A could not be measured because of its very weak binding affinity.

Table 3. EBV-EA-inducing activities of 8-decylbenzolactam-V8 (**4**) and 8-decylbenzolactone-V8 (**6**)[a]

Compound	% of EA-positive cells			
	10^{-7} M	10^{-6} M	10^{-5} M	$10^{-4.5}$ M[b]
(−)-Indolactam-V (**1**)	16.8 (2.1)[c]	33.5 (4.1)	28.9 (0.5)	
8-Decylbenzolactam-V8 (**4**)		6.7 (0.1)	11.3 (0.5)	17.6 (1.3)[d]
8-Decylbenzolactone-V8 (**6**)		0.8 (0.2)	1.9 (0.9)	5.0 (1.6)

[a]This assay was done by the method reported previously.[25,26] The cell viability exceeded 80% in all experiments except for **4** at $10^{-4.5}$ M.
[b]Data at 10^{-4} M could not be obtained because the cell viability was 0%.
[c]Standard deviation.
[d]Cell viability: 65.7%.

Table 4. Superoxide generation-inducing activities of 8-decylbenzolactam-V8 (**4**) and 8-decylbenzolactone-V8 (**6**)[a]

Compound	O_2^- generation (nmol/mL min)			
	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
(−)-Indolactam-V (**1**)	0.05 (0.07)[b]	3.19 (0.10)	3.18 (0.08)	
8-Decylbenzolactam-V8 (**4**)	0.33 (0.29)	0.31 (0.18)	3.04 (0.14)	
8-Decylbenzolactone-V8 (**6**)		0.11 (0.02)	0.20 (0.08)	0.19 (0.03)

[a]This assay was done by the method reported previously[28] with slight modification.
[b]Standard deviation.

However, it is noteworthy that **6** bound to η-C1B more selectively than **1** and **4**; the binding affinity of **6** for η-C1B was about 2-fold, 10-fold, and more than 50-fold higher than those for ε-C1B, δ- and θ-C1B, and the other PKC C1 peptides, respectively. These results suggest that relative contribution of the amide hydrogen of **1** and benzolactam-V8's to the η-C1B binding is smaller than that to the other PKC C1 peptide binding.

Compound **4** showed about 10-fold lower activities than **1** in both EBV-EA induction test and superoxide generation test. This indicates that **4** might be 10-fold weaker as a tumor promoter than **1**. However, **6** was inactive even at $10^{-4.5}$ M in the EBV-EA induction test and at 10^{-4} M in the superoxide generation test. These results also support the theory that the amide hydrogen of **1** and benzolactam-V8's plays a critical role in tumor promotion. It is recently reported that PKCα and βII are expressed in Raji B cells and that PKCβ is essential for the superoxide generation in differentiated HL-60 cells.[33,34] These data are consistent with quite weak binding abilities of **6** to the C1 peptides of PKCα and β.

In summary, we have synthesized 8-decylbenzolactone-V8 (**6**), a lactone analogue of 8-decylbenzolactam-V8 (**4**), to investigate the role of the amide hydrogen of (−)-indolactam-V (**1**) and benzolactam-V8's on PKC binding and tumor promotion. Compound **6** was far less active than either **1** or **4** in the three in vitro bioassays related to in vivo tumor promotion, indicating that the amide hydrogen of **1** and benzolactam-V8's plays a critical role in the PKC binding and tumor promotion. The PKC surrogate binding assay also revealed that the role of the amide hydrogen of **1** and benzolactam-V8's is significantly different among the PKC isozymes. The present results provide the basis for the rational design of new medicinal agents with PKC isozyme selectivity. Compound **6** might be a lead compound for a PKCη selective modulator.

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18. *epi*-Benzolactone-V8 (**15**): $[\alpha]_D$ −174.0° (c = 0.57, MeOH, 27.8 °C); UV λ_{max} (MeOH) nm (ε) 256 (5000), 207 (13,800); HR-EIMS m/z: 263.1500 (M^+, calcd for $C_{15}H_{21}NO_3$, 263.1521).
19. Nine-membered benzolactone (**16**): $[\alpha]_D$ −75.0° (c = 0.40, MeOH, 27.8 °C); UV λ_{max} (MeOH) nm (ε) 270 (2900), 235 (2500), 206 (11,500); ^{1}H NMR (500 MHz, $CDCl_3$, 0.061 M, 27 °C) δ ppm: 1.00 (3H, d, J = 6.6 Hz), 1.13 (3H, d, J = 6.7 Hz), 2.01 (1H, d, J = 7.3 Hz), 2.23 (1H, m), 2.71 (3H, s), 2.79 (1H, dd, J = 12.7, 2.7 Hz), 3.04 (1H, dd, J = 12.7, 10.4 Hz), 3.14 (1H, d, J = 9.7 Hz), 4.00 (1H, br.s), 4.17 (1H, m), 4.57 (1H, br.s), 7.15–7.24 (4H, m); HR-EIMS m/z: 263.1497 (M^+ calcd for $C_{15}H_{21}NO_3$, 263.1521).
20. Benzolactone-V8 (**5**) : $[\alpha]_D$ −124.0° (c = 0.68, MeOH, 27.8 °C); UV λ_{max} (MeOH) nm (ε) 254 (4300), 207 (14,000); HR-EIMS m/z: 263.1496 (M^+, calcd for $C_{15}H_{21}NO_3$, 263.1521).
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22. 8-Decylbenzolactone-V8 (**6**) : $[\alpha]_D$ −84.0° (c = 0.42, $CHCl_3$, 24.3 °C); UV λ_{max} (MeOH) nm (ε) 260 (2500); ^{1}H NMR (500 MHz, $CDCl_3$, 0.088 M, 27 °C) δ ppm: 0.88 (3H, t, J = 6.9 Hz), 0.97 (3H, d, J = 6.6 Hz), 1.05 (3H, d, J = 6.6 Hz), 1.23–1.31 (14H, m), 1.58 (2H, m), 2.17 (2H, m), 2.53 (2H, t, J = 7.8 Hz), 2.76 (3H, s), 2.87 (1H, dd, J = 15.8, 5.1 Hz), 3.01 (1H, dd, J = 15.8, 4.0 Hz), 3.28 (1H, d, J = 10.2 Hz), 3.69 (2H, m), 4.87 (1H, m), 6.86 (1H, s), 7.02 (2H, s); ^{13}C NMR (125 MHz, $CDCl_3$, 0.088 M, 27 °C) δ ppm: 14.12, 19.28, 19.87, 22.70, 26.68, 29.34, 29.41, 29.52, 29.62, 29.64, 31.52, 31.93, 35.27, 35.31, 36.39, 64.49, 74.66, 77.54, 126.74, 127.93, 132.17, 133.41, 139.68, 148.30, 170.68; HR-EIMS m/z: 403.3064 (M^+, calcd for $C_{25}H_{41}NO_3$, 403.3086).
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