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Cathepsin B inhibitory activities of three new phthalate derivatives isolated from seahorse, *Hippocampus Kuda* Bleeler

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ABSTRACT

Three new phthalate acid derivatives, 2,12-diethyl-11-methylhexadecyl 2-ethyl-11-methylhexadecyl phthalate (**1**), 2-ethyldecyl 2-ethylundecyl phthalate (**2**), and bis(2-ethyldodecyl) phthalate (**3**), were isolated from seahorse, *Hippocampus Kuda* Bleeler, together with a known natural analog bis(2-ethylheptyl) phthalate (**4**). The structures of these compounds were elucidated mainly by means of the comprehensive analysis of their NMR spectroscopic data. The four phthalate derivatives showed dose-dependent cathepsin B inhibitions activities with IC_{50} values of 0.13 mM (**1**), 0.21 mM (**2**), 0.18 mM (**3**), and 0.29 mM (**4**), respectively.

Seahorse belongs to *syngnathidae* of *syngnathiformes* in *Steichthyes* of vertebrate phylum. It has been widely used as a traditional medicine and/or invigorant for the treatment of erectile dysfunction (ED) due to the compellent activity like hormone from crude extract for thousands of years in the Orient. The modern pharmacological study revealed that seahorse (*Hippocampus Kuda* Bleeler) had variously bioactivities such as anti-tumor, anti-aging, and antifatigue.[1] However, the reports of chemical components responsible to these effects are rarely found so far. It greatly encouraged us to take it as our interest target for further investigation deeply.

As part of an effort to discover bioactive natural products from marine resources, we have investigated chemical constituents of *H. Kuda* Bleeler, and obtained three new natural phthalate derivatives, together with one known analogic derivative. In this paper, we report the isolation and structural elucidation of three new phthalate derivatives (**1, 2**, and **3**), and one known derivative (**4**) which was isolated from natural source for the first time. The studies on phthalate derivatives, which occurred from natural resources such as plants,[2–4] marine bacterium, marine fungi,[5–8] marine animal, and marine algae, etc.,[9–12] are increasingly becoming the focus corresponding to the variously interesting activities and mechanisms reported recently,[13–19] especially their potential mechanisms of hormone-like activity, as well as toxicity.[20–24] Furthermore, Analysis of the natural abundance ^{14}C content of the relative derivatives from two edible brown algae, supported that the phthalates can be produced naturally and accordingly.[25] Meanwhile, the evaluation of cathepsin B inhibition activities of these phthalate derivatives was also described herein.

Hippocampus Kuda Bleeler was extracted with methanol at room temperature to give the methanol extract (ME). ME was subjected to SiO_2 gel flash chromatography, followed by C-18 ODS gradient chromatography and reverse-phase HPLC, to yield four phthalate derivatives, three new compounds (**1, 2**, and **3**) and one natural-occuring known compound (**4**).

The live adults of sea horse were collected from Zhoushan Island, Zhejiang, China, in October, 2005, and freeze-dried after removing the internal organs locus in quo. It was identified as *H. Kuda* Bleeler by Professor Ming-Lu Deng (Zoologist, Changchun University of Chinese Medicine, China).

The freeze-dried *H. Kuda* Bleeler (2 kg) was ground into powder and extracted at room temperature with MeOH (3× 5 L) for 15 d, and the solvent was evaporated in vacuo to give a crude MeOH extract (138 g). The extract was subjected to silica gel flash chromatography eluted with *n*-hexane/EtOAc/MeOH (gradient), and afforded 18 main fractions. Fraction 8 (630 mg, *n*-hexane/EtOAc, 5:1) was chromatographed over ODS by elution with MeOH/H_2O to yield compounds **1–4**, respectively. The isolates were further purified by HPLC (Dionex, ODS, MeOH) utilizing a 40 min gradient program of 50–100% MeOH in H_2O to furnish compound **1** (15.6 mg), compound **2** (21.5 mg), compound **3** (20.2 mg), and compound (**4**, 17.8 mg).

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12-Diethyl-11-methylhexadecyl 2-ethyl-11-methylhexadecyl phthalate (**1**): yellowish oil; $[\alpha]_D^{20}$ 0.1° (*c* 2.0, MeOH); IR (neat) ν_{max} 2957, 2924, 2855, 1720, 1602, 1575, 1485, 1264, 1115, 1072, 1033, 965, 798, 741 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ε) 274 (2.39) nm; HR-ESIMS *m/z* 726.6547 $[M]^+$ (calcd for $C_{48}H_{86}O_4$, 726.6526, Δ +2.1 mu), LREIMS *m/z* (rel. int.) 726 $[M]^+$ (2), 474 (2), 419 (2), 293 (17), 279 (8), 249 (2), 177 (3), 167 (21), 149 (91),127 (9), 113 (2), 98 (10), 85 (36), 71 (44), 57 (100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (2H, dd, *J* = 8.8, 2.2 Hz, H-3, 6), 7.52 (2H, dd, *J* = 8.8, 2.2 Hz, H-4, 5), 4.27, 4.21 (4H, t, *J* = 6.3 Hz, H_2-1′, 1″), 1.68 (4H, m, H-2′, 2″, 11′, 11″), 1.45 (1H, m, H-12″), 1.33 (2H, m, H_2-15′), 1.32 (2H, m, H_2-15″), 1.29 (36H, m, H_2-4′, 4″, 5′, 5″, 6′, 6″, 7′, 7″, 8′, 8″, 9′, 9″, 13′, 14′, 14″, 17′, 17″, 20″), 1.25 (12H, m, H_2-3′, 3″, 10′, 10″, 12′, 13″), 0.90 (21H, m, H3-16′, 16″, 18′, 18″, 19′, 19″, 21″); ^{13}C NMR (100 MHz, $CDCl_3$) data,[26] see Table 1.

2-Ethyldecyl 2-ethylundecyl phthalate (**2**): Yellowish oil; $[\alpha]_D^{20}$ +0.0030 (*c* 1.2, $CHCl_3$); IR (neat) ν_{max} 2924, 1721, 1604, 1571, 1482, 1264, 1113, 1070, 1033, 951, 748, 701 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ε) 276 (2.95) nm; HR-ESIMS *m/z* 516.4168 $[M]^+$ (calcd for $C_{33}H_{56}O_4$ 516.4179, Δ −1.1 mmu), LREIMS *m/z* (rel. int.) 516 $[M]^+$ (1), 491 (1), 429 (2), 355 (3), 334 (3), 279 (5), 167 (23), 149 (100), 113 (7), 97 (11), 84 (20), 71 (42), 57 (74); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (1H, dd, *J* = 8.8, 2.2 Hz, H-3, 6), 7.52 (2H, dd, *J* = 8.8, 2.2 Hz, H-4, 5), 4.21 (4H, t, *J* = 6.2 Hz, H_2-1′, 1″), 1.66 (4H, m, H-2′, 2″), 1.25 (26H, m, H_2-3′, 3″, 4′, 4″, 5′, 5″, 6′, 6″, 7′, 8′, 7″, 9′, 8″), 1.30 (4H, m, H_2-10′, 9″), 0.87 (6H, m, H_3-11′, 10″), 1.39 (4H, m, H_2-12′, 11″), 0.89 (6H, m, H_3-13′, 12″), ^{13}C NMR (100 MHz, $CDCl_3$) data,[26] see Table 1.

Bis(2-ethyldodecyl) phthalate (**3**): Yellowish oil; $[\alpha]_D^{20}$ 0° (*c* 0.5, MeOH)); IR (neat) ν_{max} 2920, 1720, 1602, 1577, 1482, 1461, 1379, 1267, 1120, 1067, 1035; 956, 741, 702 cm^{-1}; HR-ESIMS *m/z* 558.4653 $[M]^+$ (calcd for $C_{36}H_{62}O_4$ 558.4686, Δ −3.3 mmu), LREIMS *m/z* (rel. int.) 558 $[M]^+$ (5), 279 (8), 167 (32), 149 (100), 123 (3), 97 (11), 83 (19), 71 (45), 57 (72); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (2H, dd, *J* = 8.8, 2.2 Hz, H-3, 6), 7.53 (2H, dd, *J* = 8.8, 2.2 Hz, H-4, 5), 4.21 (4H, t, *J* = 6.2 Hz, H_2-1′, 1″), 2.04 (2H, m, H-2′, 2″), 1.25 (20H, m, H_2-3′, 3″, 4′, 4″, 5′, 5″, 7′, 7″, 8′, 8″), 1.29 (12H, m, H_2-6′, 6″, 9′, 9″, 10′, 10′″), 1.31 (4H, m, H2-11′, 11″), 0.87 (6H, m, H_3-12′, 12″), 1.60 (4H, m, H_2-13′, 13″), 0.90 (6H, m, H_3-14′, 14″); ^{13}C NMR (100 MHz, $CDCl_3$) data,[26] see Table 1.

Bis(2-ethylheptyl) phthalate (**4**): Yellowish oil; $[\alpha]_D^{20}$ 0° (*c* 0.5, MeOH)); IR (neat) ν_{max} 2924, 1723, 1600, 1577, 1488, 1462, 1379, 1267, 1118, 1071, 1037; 956, 741, 702 cm^{-1}; LREIMS *m/z* (rel. int.) 418 $[M]^+$ (5), 279 (7), 167 (22), 149 (100), 113 (11), 83 (19), 71 (35), 57 (83); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (2H, dd, *J* = 8.8, 2.2 Hz, H-3, 6), 7.53 (2H, dd, *J* = 8.8, 2.2 Hz, H-4, 5), 4.21 (4H, t, *J* = 6.2 Hz, H_2-1′, 1″), 1.65 (4H, m, H_2-2′, 2″), 1.25 (4H, m, H_2-3′, 3″), 1.29 (8H, m, H_2- 4′, 4″, 5′, 5″), 1.31 (4H, m, H_2-6′, 6″), 0.89 (6H, m, H_3-7′, 7″), 1.40 (4H, m, H_2-8′, 8″), 0.90 (6H, m, H_3-9′, 9″); ^{13}C NMR (100 MHz, $CDCl_3$) data,[26] see Table 1.

Table 1
^{13}C data for compounds **1–4** in $CDCl_3$

Position	1	2	3	4
1	167.7 (s)	167.8 (s)	167.8 (s)	167.8 (s)
2	132.3 (s)	132.4 (s)	132.4 (s)	132.4 (s)
3	128.8 (d)	128.8 (d)	128.8 (d)	128.8 (d)
4	130.9 (d)	130.9 (d)	130.9 (d)	130.9 (d)
5	130.9 (d)	130.9 (d)	130.9 (d)	130.9 (d)
6	128.8 (d)	128.8 (d)	128.8 (d)	128.8 (d)
7	132.3 (s)	132.4 (s)	132.4 (s)	132.4 (s)
8	167.7 (s)	167.8 (s)	167.8 (s)	167.8 (s)
1′	66.2 (t)	68.1 (t)	68.1 (t)	68.1 (t)
2′	38.7 (d)	38.7 (d)	38.7 (d)	38.7 (d)
3′	31.9 (t)	30.3 (t)	30.3 (t)	30.3 (t)
4′	28.6 (t)	28.9 (t)	28.9 (t)	28.9 (t)
5′	30.3 (t)	29.7 (t)	29.7 (t)	29.7 (t)
6′	29.7 (t)	29.7 (t)	29.7 (t)	22.9 (d)
7′	29.7 (t)	29.7 (t)	29.7 (t)	14.0 (q)
8′	29.7 (t)	29.3 (t)	29.7 (t)	23.7 (t)
9′	27.0 (t)	31.9 (t)	29.4 (t)	10.9 (q)
10′	34.3 (t)	22.9 (t)	31.9 (t)	
11′	33.1(d)	14.2 (q)	22.7 (t)	
12′	36.6 (t)	23.7 (t)	14.0 (q)	
13′	26.8 (t)	10.9 (q)	23.7 (t)	
14′	29.2 (t)		11.0 (q)	
15′	22.7 (t)			
16′	14.1 (q)			
17′	23.7 (t)			
18′	11.0 (q)			
19′	19.6 (q)			
1″	66.2 (t)	68.1 (t)	68.1 (t)	68.1 (t)
2″	38.7 (d)	38.7 (d)	38.7 (d)	38.7 (d)
3″	31.9 (t)	30.3 (t)	30.3 (t)	30.3 (t)
4″	28.9 (t)	28.9 (t)	28.9 (t)	28.9 (t)
5″	30.3 (t)	29.7 (t)	29.7 (t)	29.7 (t)
6″	29.7 (t)	29.7 (t)	29.7 (t)	22.9 (d)
7″	29.7 (t)	29.3 (t)	29.7 (t)	14.0 (q)
8″	29.7 (t)	31.8 (t)	29.7 (t)	23.7 (t)
9″	27.5 (t)	22.6 (t)	29.4 (t)	10.9 (q)
10″	36.5 (t)	14.1 (q)	31.9 (t)	
11″	39.4 (d)	23.7 (t)	22.9 (t)	
12″	41.9 (d)	10.9 (q)	14.1 (q)	
13″	32.5 (t)		23.7 (t)	
14″	32.7 (t)		11.0 (q)	
15″	23.0 (t)			
16″	14.2 (q)			
17″	23.7 (t)			
18″	11.4 (q)			
19″	19.1 (q)			
20″	26.1 (t)			
21″	12.2 (q)			

Recorded in $CDCl_3$ at 400 MHz (^{1}H) and 100 MHz (^{13}C).

The molecular formula of compound (**1**) was determined to be $C_{48}H_{86}O_4$ (6 deg of unsaturation) by HR-ESIMS analysis [*m/z* 726.6547, Δ +2.1 mmu], which was supported by ^{1}H, ^{13}C, and ^{13}C DEPT NMR data. The IR spectrum of **1** showed absorptions for alkane (2924 cm^{-1}, C–H stretch), carbonyl (1720 cm^{-1}, C=O), aromatic (1602, 1575, 1485, 741 cm^{-1}, C=C), ester (1264, 1115, 1072, 1033 cm^{-1}, C–O) groups. The UV spectrum of **1** showed the presence of conjugated system [274 nm (log ε = 2.39)]. Analysis of the ^{1}H, ^{13}C, DEPT, and HMQC NMR data of **1** revealed the presence of seven methyl groups, 28 methylene units (two *O*-bearing methylene), four sp^2 aromatic carbons, five methines, two sp^2 quaternary carbons, and two carbonyl carbons. An AA′BB′ system existed in ^{1}H NMR spectrum at δ 7.70 (2H, dd, *J* = 8.8, 2.2 Hz) and 7.52 (2H, dd, *J* = 8.8, 2.2 Hz). These data accounted for all required the compound to have one benzene ring (three double bonds and one ring, 4 deg of unsaturation), and side chain with two carbonyl groups (2 deg of unsaturation). The NMR data for **1** were characteristic of a disubstituted phenyl structure. Analysis of ^{1}H and ^{13}C NMR chemical shifts and the coupling constants observed for four aromatic protons led to the identification of one *ortho*-substituted aromatic ring. These assignments were further confirmed by relevant HMBC correlations of **1**. Two aromatic protons (δ 7.70) present in **1** showed HMBC correlations with C-1, C-2, and C-4, respectively, indicating that two carbonyl carbons were directly connected to aromatic sp^2 quaternary carbons (C-2 and C-7). Due to the ^{13}C chemical shift of these two carbonyl carbons, and ^{1}H and ^{13}C NMR data of two *O*-bearing methylene units, the structure of compound (**1**) was suggested to be a phthalate derivative, supported by HMBC correlations of H-1′ and H-1″ with C-1 and C-8, respectively. The connections of the two side-chain moieties were completely assigned by HMBC and COSY correlations. On the basis of these data, the planar structure of compound (**1**) was established as 2, 12-diethyl-11-methylhexadecyl 2-ethyl-11-methylhexadecyl phthalate. The structure and key correlations from HMBC and COSY spectra of compound (**1**) were described in Figures 1 and 2, respectively.

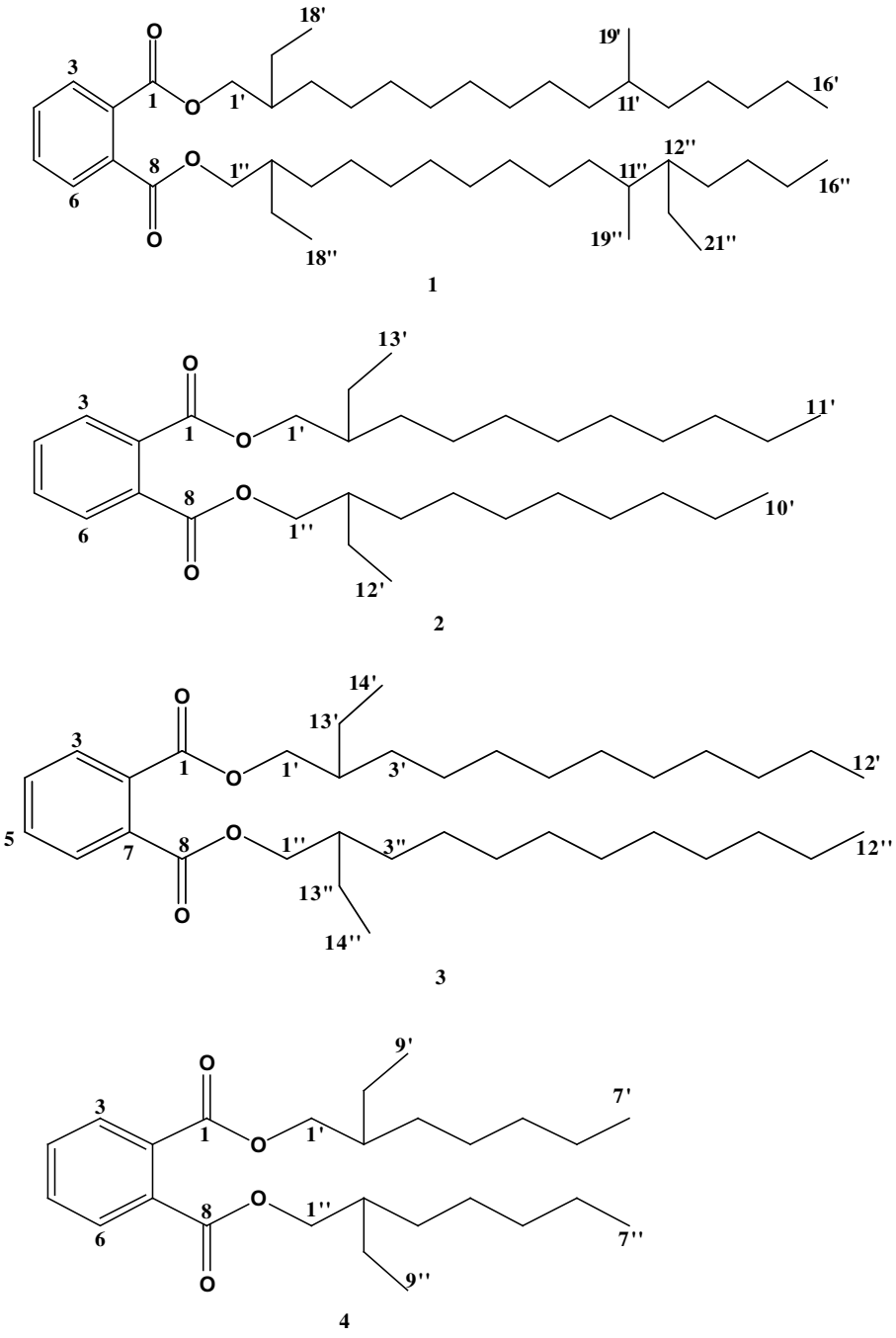

Figure 1. Chemical structures of phthalate derivatives.

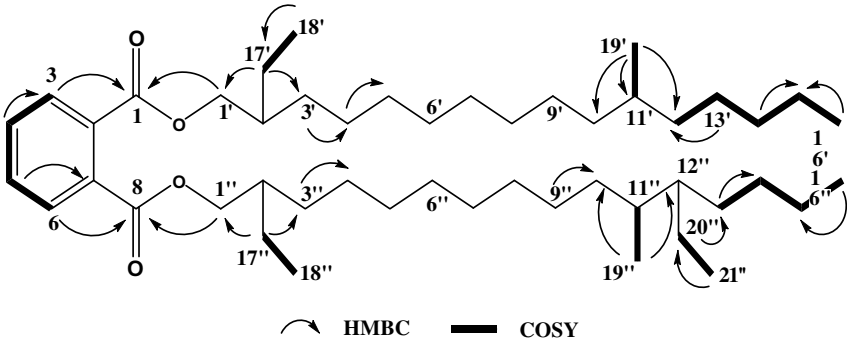

Figure 2. Key HMBC and COSY correlations of compound **1**.

The relative stereochemistry was determined by ^{1}H–^{1}H NOESY experiment, the correlations from H-2′/H-1′, 3′; H-2″/H-1″, 3″; H-11′/H-10′, 12′, 19′; H-11″/10″,12″, 19″; H-12″/H-11″,13″ in NOESY spectrum were observed indicating the same equator position for those protons, however, no correlations were revealed for H-2′/H-17′; H-2″/H-17″; and H-12″/H-20″. Therefore, the stereo locations of the three ethyl groups at C-2′, 2″, and 12″ were different from the two alkyl side chains. The four chiral carbons (C-2′, 2″, 11′, 11″) existed in compound (**1**) were symmetric, the ethyl group (*R*, C-12″) attached to the other chiral carbon (C-12″) led to the molecular asymmetry, which was also supported by optical rotation data, namely, $[\alpha]_D^{20}$ 0.1° (*c* 2.0, MeOH).

The molecular formula of compound (**2**) was determined to be $C_{33}H_{56}O_4$ (6 deg of unsaturation) by HR-ESIMS analysis (*m/z* 516.4168 M^+, calcd for $C_{33}H_{56}O_4$ 516.4179, Δ −1.1 mmu), and this conclusion was supported by 1D NMR data. The IR spectrum of **2** showed functional groups such as alkane (2924 cm^{-1}, C–H stretch), carbonyl (1721 cm^{-1}, C=O), aromatic (1604, 1571, 1482, 748 cm^{-1}, C=C), and ester (1264, 1113, 1070, 1033 cm^{-1}, C–O), respectively. The UV spectrum of **2** revealed the absorption at 276 nm ($\log\varepsilon$ = 2.95) indicating presence of conjugated system. Analysis of the NMR spectra including 1D and 2D showed the presence of four methyl groups, nineteen methylene units (two *O*-bearing methylenes), four sp^2 aromatic carbons, two methines, two sp^2 quaternary Carbons, and two carbonyl carbons. ^{1}H NMR spectrum showed an AA′BB′ system at δ 7.70 and 7.52 ppm. These data accounted for all required the compound to have one benzene ring (three double bonds and one ring, 4 deg of unsaturation), and side chain with two carbonyl groups (2 deg of unsaturation). The NMR data for **2** were characteristic of a disubstituted phenyl structure. Analysis of ^{1}H and ^{13}C NMR chemical shifts and the *J* values observed for four aromatic protons led to the identification of one *ortho*-substituted aromatic ring. These assignments were further confirmed by relevant HMBC correlations. Two aromatic protons (δ 7.70) present in **2** showed HMBC correlations with C-1, C-2, and C-4, respectively, indicating that two carbonyl carbons were directly connected to aromatic sp^2 quaternary carbons (C-2 and C-7). Due to the ^{13}C chemical shifts of these two carbonyl carbons as well as ^{1}H and ^{13}C NMR data of two *O*-bearing methylene units, the structure of compound (**2**) was suggested to be a phthalate derivative, supported by HMBC correlations of H-1′ and H-1″ with C-1 and C-8. The connections of the two side-chain moieties were completely assigned by HMBC and COSY correlations. The two chiral carbons (C-2′ and 2″) existed in compound (**2**) were symmetric, the two side chains with one methylene unit deference led to the molecular asymmetry, and ^{13}C NMR data at δ 31.9 (C-9′) and 31.8 (C-8″), 22.9 (C-10′) and 22.6 (C-9″) as well as the two terminal methyl carbons at 14.2 (C-11′) and 14.1 (C-10″). Therefore, the comprehensive analyses of MS, 1D and 2D NMR data including NOESY completely led to the elucidation of compound (**2**) as 2-ethyldecyl 2-ethylundecyl phthalate. The chemical structure of compound (**2**) was described in Figure 1.

Compound **3** was isolated as yellowish oil. The molecular formula was established as $C_{36}H_{62}O_4$ by HR-EIMS spectral data, which showed a $[M]^+$ peak at *m/z* 558.8821. This formula was confirmed by 1D NMR suggesting the presence of a phthalate derivative. The fragmentation pattern of the mass spectrum in MS exhibited three fingerprint ion signals at *m/z* 279.20, 167.10, and 149.15 corresponding to the skeleton of phthalate derivatives. The molecular formula also revealed the six unsaturated degrees produced from two carbonyl groups, one benzoic ring. The IR spectrum of compound **3** supported this result by the strong absorptions of carbonyl (1723 cm^{-1}, C=O), aromatic (1600, 1577, 1488, 741 cm^{-1}, C=C), ester (1267, 1118, 1071, 1037 cm^{-1}, C–O), and aromatic (956, 741, 702 cm^{-1}, C–H, out-of plane bend) groups. The ^{1}H NMR spectrum of **3** showed a typical AA′BB′ system at δ 7.70 and 7.53 ppm. The chemical shifts and coupling constants of these aromatic protons implied that the compound must have an *ortho*-disubstituted benzene ring bearing the same substituents in both side chains. The NMR (1D and 2D) signals indicated two carbonyl carbons, four aromatic methine, two *O*-bearing methylene, four methyl, two alkyl methine, and twenty alkyl methylene groups. The HMBC and COSY spectra of compound **3** revealed the key correlations, H-4/5 to C-2/7, 3/6, 4/5; H-3/6 to C-1/8; H-1′/1″ to C-1/8, C-2′/2″; H-13′/13″ to C-1′/1″ and 3′/3″. The stereochemistry was completed by NOESY experiment due to the presence of chiral carbons. Accordingly, the structure of compound **3** was clearly elucidated as a new bis(2-ethyldodecyl) phthalate, which was described in Figure 1.

Compound **4** had the molecular formula $C_{26}H_{42}O_4$ as determined from LREIMS, ^{1}H, ^{13}C, and ^{13}C DEPT spectral data. The six unsaturated degrees were from two carbonyl groups, three aromatic double bonds, and one aromatic ring. The IR spectrum of compound **4** suggested the presence of alkane (2924 cm^{-1}, C–H stretch), carbonyl (1723 cm^{-1}, C=O), aromatic (1600, 15771488, 741 cm^{-1}, C=C), methylene (1462 cm^{-1}, –CH_2 bend), methyl (1379 cm^{-1}, –CH_3 bend), ester (1267, 1118, 1071, 1037 cm^{-1}, C–O), and aromatic (956, 741, 702 cm^{-1}, C–H, out-of plane bend) groups. The ^{1}H NMR spectrum of **4** showed a typical AA′BB′ system at δ 7.70 and 7.53 ppm. The chemical shifts and coupling constants of these aromatic protons implied that the compound must have an *ortho*-disubstituted benzene ring bearing the same substituent in both positions. The ^{13}C, DEPT, and HMQC NMR signals indicated the presence of two carbonyl carbons, four aromatic methine, two *O*-bearing methylene, four methyl, two alkyl methane, and ten alkyl methylene groups. The HMBC and COSY spectra of compound **4** revealed the key correlations, H-4/5 to C-2/7, 3/6, 4/5; H-3/6 to C-1/8; H-1′/1″ to C-1/8; H-8′/8″ to C-1′/1″, 3′/3″; H-7′/7″ to C-5′/5″, 6′/6″; H-9′/9″ to C-8′/8″, 2′/2″; and H-1′/1″ to H-2′/2″. Finally, the structure of compound **4** was assigned as a known bis(2-ethylheptyl) phthalate,[27] see Figure 1.

The lysosomal cysteine proteases cathepsin B (EC 3.4.22.1) is a unique cysteine member showing dual roles as both endopeptidase and exopeptidase activities due to the presence of the occluding loop in structure.[28] Inhibitors of cathepsin B include endogenous inhibitors such as the cystatin superfamily, low molecular weight natural, and chemical synthesis inhibitors. Meanwhile, cathepsin B reveals a role not only in intracellular protein catabolism; but also in hormone activation and processing of antigens in immune response. However, over-expression and mislocation in cell membrane of cathepsin B can be donated to some pathological processes that include cancer and neurogenerative disorders.[29–31] The effective inhibition of cathepsin B can decrease the severity of joint inflammation and to reduce the destruction of particular tissues in the rat model of antigen adjuvant-induced arthritis. Therefore, cathepsin B inhibitors are expected to be useful for the treatment of inflammatory joint disease, invasion of cancer, and other diseases related to cathepsin B disorder. In brief, to search for new cathepsin B inhibitors, especially reversible non-peptidal inhibitors, should be given increased attention.

Cathepsin B inhibitory assay of these phthalate derivatives was carried out in 96-well black plate by the method of Barrett et al.[27] with some modifications.[32] The four phthalate derivatives (**1**–**4**) showed dose-dependent cathepsin B inhibitions activities with 0.13 mM (**1**), 0.21 mM (**2**), 0.18 mM (**3**), and 0.29 mM (**4**), respectively, see Figure 3. Among these compounds, compound **1** and **3** showed more interesting activity than others.

In order to prove the non-contamination process during the sample collection (freshly live adults of sea horse were collected and freeze-dried after removing the internal organs locus in quo.), extraction, isolation, and purification (non-plastic wares), a blank control without seahorse sample were carried out at the

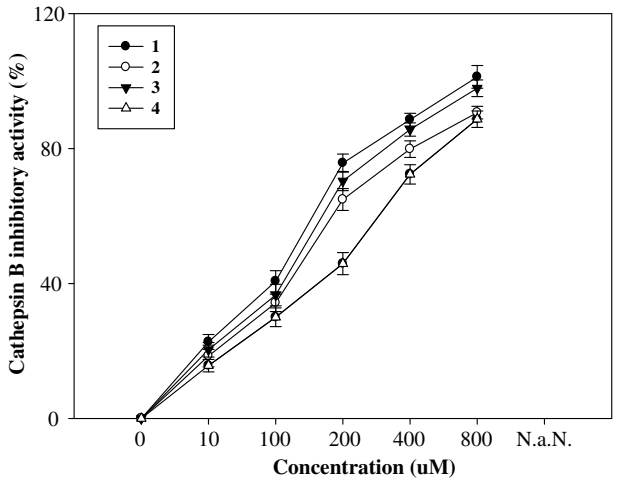

Figure 3. Dose-dependent inhibitions of cathepsin B by phthalate derivatives. The values are means ± SE.

same time, and the result of TLC and HPLC analysis indicated that those phthalates derivatives were naturally produced from sea-horse indeed.[9] In latest investigations, phthalates derives showed the hormone-like activity among their various bioactivities. Because of the consideration of the treatment for erectile dysfunction (ED) using sea horse as a traditional medicine with a long history, these compounds should be more interesting and urgent for the further study, especially the three new compounds (**1**–**3**) and compound (**4**) with the detail elucidation by NMR spectra technology.

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References and notes

1. Zhang, N.; Xu, B.; Mou, C. Y.; Yang, W. L.; Wei, J. W.; Liang, L.; Zhu, J.; Du, J.; Wu, X.; Ye, L.; Fu, Z.; Lu, Y.; Lin, J.; Sun, Z.; Su, J.; Dong, M.; Xu, A. *FBES Lett.* **2003**, *550*, 124.
2. Lee, K. H. J.; Kim, H.; Lim, D. S.; Kim, C. H. *J. Pharm. Pharmacol.* **2000**, *52*, 593.
3. Ram, K. V.; Madan, M. G. *Phytochemistry* **1988**, *27*, 459.
4. Jocelyn, P. J. R.; Belanger, J. K.; Jankowski, K. *J. Nat. Prod.* **1981**, *44*, 490.
5. Al-Bari, M. A.; Bhuiyan, M. S.; Flores, M. E.; Petrosyan, P.; Garcia-Varela, M.; Islam, M. A. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1973.
6. Amade, P.; Mallea, M.; Bouaicha, N. *J. Antibiot.* **1994**, *47*, 201.
7. Chen, C. Y. *Water Res.* **2004**, *38*, 1014.
8. Hoang, V. L. T.; Li, Y.; Kim, S. K. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2083.
9. Noguchi, T.; Ikawa, M.; Uebel, J. J.; Andersen, K. K. Walter de Gruyter & Co.: New York, 1979, pp. 711–718.
10. Sastry, V. M. V. S.; Rao, G. R. K. *J. Appl. Phycol.* **1995**, *7*, 185.
11. Stefanov, K.; Konaklieva, M.; Brechany, E. Y.; Christie, W. W. *Phytochemistry* **1988**, *27*, 3495.
12. Rameshthangam, P.; Ramasamy, P. *Virus Res.* **2007**, *126*, 38.
13. Pereira, C.; Mapuskar, K.; Rao, C. V. *Environ. Toxicol. Pharmacol.* **2007**, *23*, 319.
14. Tavridou, A.; Manolopoulos, V. G. *Eur. J. Pharm.* **2004**, *505*, 213.
15. Takeuchi, S.; Iida, M.; Kobayashi, S.; Jin, K.; Matsuda, T.; Kojima, H. *Toxicology* **2005**, *210*, 223.
16. Borch, J.; Metzdorff, S. B.; Vinggaard, A. M.; Brokken, L.; Dalgaard, M. *Toxicology* **2006**, *223*, 144.
17. Barse, A. V.; Chakrabarti, T.; Ghosh, T. K.; Pal, A. k.; Jadhao, S. B. *Biol. Chem. Physiol.* **2007**, *88*, 36.
18. Larsen, S. T.; Nielsen, G. D. *Toxicol. Lett.* **2007**, *170*, 223.
19. Mapuskar, K.; Pereira, C.; Rao, C. V. *Pestic. Biochem. Physiol.* **2007**, *87*, 156.
20. Jiang, J.; Ma, L.; Yuan, L.; Wang, X.; Zhang, W. *Toxicology* **2007**, *232*, 286.
21. Andrade, A. J. M.; Grande, S. W. C.; Talsness, E.; Grote, K.; Golombiewski, A.; Sternerkock, A.; Chahoud, I. *Toxicology* **2006**, *225*, 64.
22. Stroheker, T.; Cabaton, N.; Nourdin, G.; Regnier, J.; Lhuguenot, J.; Chagnon, M. *Toxicology* **2005**, *208*, 115.
23. Stroheker, T.; Regnier, J. F.; Lassurguere, J.; Chagnon, M. C. *Food Chem. Toxicol.* **2006**, *44*, 2064.
24. Namikoshi, M.; Fujiwara, T.; Nishikawa, T.; Ukai, K. *Mar. Drugs* **2006**, *4*, 290.
25. Beher, A.; Urschey, M.; Brehme, V. A. *Green Chem.* **2003**, *5*, 198.
26. Melting points were determined on an Electrothermal model IA 9100 micro-melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer model 341 polarimeter. IR spectra were recorded on a Bruker FT-IR model IFS-88 spectrometer. MS spectra were obtained on a JEOL JMS-700 spectrometer. UV/vis spectra were measured on a Hitachi U-2001 UV/vis spectrometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Japan), using $CDCl_3$ (0.01% TMS) solvent peaks (δ 7.26 ppm in ^{1}H and δ 77.6 ppm in ^{13}C NMR) as an internal reference standard. For some signals, the chemical shifts approximated third decimal place. This is to distinguish between signals of very close value but which could nevertheless be clearly differentiated by visual inspection of the spectra. MS spectra were obtained on a JEOL JMS-700 spectrometer (JEOL, Japan). Extraction of seahorse was performed using Extraction Unit (Dongwon Scientific Co., Korea). Column chromatography was carried out by silica gel 60 (230–400 mesh, Merck, Germany).
27. Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. *Biochem. J.* **1982**, *201*, 189.
28. Mort, J. S.; Buttle, D. J. *Int. J. Biochem. Cell Biol.* **1997**, *29*, 715.
29. Leung-Toung, R.; Li, W.; Tam, T. F.; Karimian, K. *Curr. Med. Chem.* **2002**, *9*, 979.
30. Szpaderska, A. M.; Frankfater, A. *Cancer Res.* **2001**, *61*, 3493.
31. Petanceska, S.; Burke, S.; Watson, S. J.; Devi, L. *Neuroscience* **1994**, *59*, 729.
32. Cathepsin B inhibitory assay was performed in 96-well black plate by the method of Barrett et al.[26] with some modifications. Each well contains 12.5 μl of a 0.4 M sodium potassium phosphate buffer (pH 6.0) containing 8 mM dithiothreitol (DTT) and 4 mM EDTA, 12.5 μl of compound solution (dissolved in methanol). After 10 min at 37 °C, 12.5 μl of 20 μM Z-Arg-Arg-MCA in water was added to start the reaction. The reaction was stopped after 20 min by 50 μl of 100 mM sodium monochloroacetate in 100 mM sodium acetate buffer (pH 4.3). Methanol was used for control value. The fluorescence of 4-methyl-coumary-7-amide released was measured at Ex 360 nm and Em 465 nm with GENios® microplate reader (Tecan Austria GmbH, Austria). All experiments were carried out at least in triplicate and results are reported as means ± standard deviation.