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Graphical Abstract

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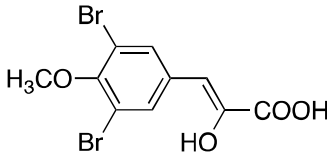

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ApoE secretion modulatingbromotyrosinederivative from the Australian marine sponge *Callyspongia*sp.

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ABSTRACT

High Throughput Screening of a pre-fractionated natural product library identified 11 active fractions showing ApoE modulation activity.Mass-directed fractionation of oneactivecrude extract from the Australian marine sponge *Callyspongia* sp. Resulted in the isolation of 13 metabolites, including three new bromotyrosine derivatives, callyspongic acid (**1**), 3,5-dibromo-4-methoxyphenylpyruvic acid (**2**), *N*-acetyl-3-bromo-4-hydroxyphenylethamine (**3**), and ten known compounds (**4**-**13**). The structure elucidation of compounds **1**-**3** was based on their 1D and 2D NMR and MS spectroscopic data. 3,5-dibromo-4-methoxyphenylpyruvic acid (**2**) showed weak activity in increasing the apolipoprotein E secretion from human CCF-STTG1 cellsat the concentration of 40 μM.

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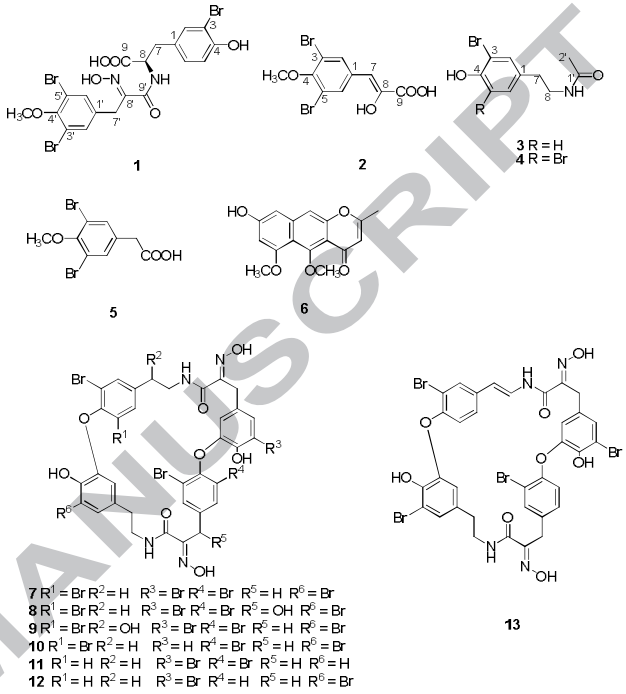
Alzheimer's disease (AD) has emerged as the most prevalent form of late-life mental disorder in humans and is typified by deposition of β-amyloid (Aβ) with the brain.[1] Aβ is a neurotoxic peptide and the accumulation of Aβ leads to its deposition into plaques and the launching of a pathologic cascade that ultimately leads to neuronal death.[1] An impaired ability to clear Aβ peptides from the brain, rather than increased production of Aβ peptides, is thought to underlie most cases of late-onset AD.[2]

Apolipoprotein E (ApoE) is the major apolipoprotein in the central nervous system that plays a central role in cholesterol transport.[3] In the brain, ApoE is mostly produced by astrocytes, and some of ApoE binds to specific neuronal receptors for cholesterol uptake.[3] A previous study suggested that increased levels of highly lapidated ApoE could facilitate the clearance of Aβ peptides,[4, 5] implying that the interference of ApoE secretion could potentially lead to the treatment of Alzheimer's disease. Bexarotene is the only reported compound used clinically that is known to activate ApoE expression.[5]

In our ongoing search for new lead compounds for neurodegenerative disorders, a drug discovery program was initiated to identify natural products as ApoE modulators. A high throughput screening method was developed and used to screen EskitisInstitute's pre-fractionated natural product library of 102,432 fractions for their ability to modulate ApoE secretion from the human derived astrocytoma cell line CCF-STTG1, used extensively in Alzheimer research.[6] Eleven fractions were identified with concentration dependent activity for ApoE enhancement over background levels, albeit at high concentrations. One of the 11 fractions was derived from an extract of the Australian sponge *Callyspongia* sp.(-)-LRESIMS of the active fraction showed molecular ions at *m/z*349/351/353, 321/323/325, which were predicted to correspond to bioactive natural products. Mass-directed fractionation of the CH_2Cl_2/MeOH extract led to the isolation of 13 compounds, including three new bromotyrosine derivatives, callyspongic acid (**1**), 3,5-dibromo-4-methoxyphenylpyruvic acid (**2**),and *N*-acetyl-3-bromo-4-hydroxylphenylethamine (**3**), together with ten known natural products, *N*-acetyl-3,5-dibromo-4-hydroxyl phenylethamine (**4**),[7] 3,5-dibromo-4-methoxyphenylacetic acid(**5**),[8] comantherin (**6**),[9] bastadin 6 (**7**),[10] bastadin 7 (**8**),[10] bastadin 8 (**9**),[11] bastadin 9 (**10**),[11] bastadin 16 (**11**),[12] bastadin 18 (**12**),[13] and bastadin 24 (**13**).[14] Herein we report the isolation and structure elucidation of callyspongic acid (**1**), 3,5-dibromo-4-methoxyphenylpyruvic acid (**2**),and*N*-acetyl-3-bromo-4-hydroxylphenylethamine (**3**), as well as the ApoE modulation activities of compounds **1** -**13**.

The freeze-dried and ground marine sponge (20.0 g) was sequentially extracted with *n*-hexane, CH_2Cl_2 and MeOH. The CH_2Cl_2 and MeOH extracts were combined and chromatographed on reverse phase C_{18} flash column, eluting with 10% MeOH/H_2O, 30% MeOH/H_2O, 50% MeOH/H_2O, and 100% MeOH. The 10% MeOH/H_2O was fractionatedusing reversephase C_{18}-bonded silica HPLC (MeOH/H_2O/0.1% TFA) to yield 60 fractions. Fractions 22-25 and 29 contained the ions of interest, and yielded3,5-dibromo-4-methoxyphenylpyruvic acid(**2**, 2.0 mg, 0.01% dry wt), and 3,5-dibromo-4-methoxyphenylacetic acid(**5**, 0.6 mg, 0.003% dry wt), respectively. Purification of fraction 34 with a Luna C_{18} semi-preparative HPLC column yielded callyspongic acid (**1**, 0.2 mg, 0.001% dry wt). The 30% MeOH/H_2Ofraction was further purified by reversephase C_{18}-bonded silica HPLC (MeOH/H_2O/0.1% TFA) to yield 60 fractions. Fractions 16, 34 and 46 resulted in pure compounds *N*-acetyl-3-bromo-4-hydroxylphenylethamine(**3**, 0.4 mg, 0.002% dry wt), *N*-acetyl-3,5-dibromo-4-hydroxyl phenylethamine(**4**, 0.8 mg, 0.004% dry wt), and comantherin(**6**, 1.0 mg, 0.005% dry wt). Further purification of the fractions 33-36 with a Luna C_{18}semi-preparative column yielded 3,5-dibromo-4-methoxyphenyl- aceticacid(**6**, 0.8 mg, 0.004% dry wt), bastadin18(**12**, 0.4 mg, 0.002% dry wt), and bastadin 24(**13**, 0.4 mg, 0.002% dry wt). Repeated chromatography of 50% MeOH/H_2O fraction on reverse phase C_{18}-bonded HPLC and Luna C_{18} semi-preparative HPLC yieldedbastadin 6(**7**, 0.4 mg, 0.002%



dry wt), bastadin 7

(**8**, 0.3 mg, 0.0015% dry wt), bastadin 8 (**9**, 0.50 mg, 0.0025% dry wt), bastadin 9 (**10**, 0.30 mg, 0.0015% dry wt), andbastadin 16 (**11**, 0.6 mg, 0.003% dry wt).

Callyspongic acid (**1**)[15] was obtained as a brown amorphous powder. The cluster of four isotopic ions at *m/z*607/609/611/613 (1:3:3:1) in (+)-LRESIMS indicated the presence of three bromine atoms in the molecule. The molecular formula, $C_{19}H_{17}Br_3N_2O_6$, was established on the basis of HRESIMS (*m/z*628.8529 $[M+Na]^+$, calcd628.8532 for $C_{19}H_{17}{}^{79}Br_3N_2O_6Na$), with 11degrees of unsaturation. The ^{1}Hand gHSQCNMRdata analysis suggested thatcompound **1**contained three exchangeable protons (δ_H 12.11, s; 9.98, s;and 7.87, d, J = 7.9 Hz), fivearomatic protons (δ_H 7.40, s, 2H;7.23, d, J = 2.1 Hz, 1H;6.92, dd, J = 8.3, 2.1 Hz, 1H; and 6.79, d, J = 8.3 Hz, 1H), one methine (δ_H 4.44, m), two methylenes (δ_H 3.73, d, J = 14.2 Hz, H-7'a; 3.70, d, J = 14.2 Hz, H-7'b; 3.00, dd, J = 5.0, 14.0 Hz, H-7a; and 2.95, dd, J = 8.2, 14.0 Hz, H-7b), and one methoxy (δ_H 3.74, s). The gCOSY correlation data (Fig. 1) established a 1,3,4-trisubstituted benzene moiety and an ethylamine group: NH-CH-CH_2.gHMBC experiment revealed the correlations from theexchangeable proton (δ_H 9.98) tothree aromatic carbons (δ_C 109.4, C-3;153.3, C-4; and 116.6, C-5), suggesting that the hydroxyl group was attached to the C-4 of the 1,3,4-trisubstituted benzene moiety. gHMBC correlations from the methylene (δ_H 3.00,H-7a; 2.95, H-7b)in the ethylamine group to the three aromatic carbons (δ_C 130.1, C-1; 133.7, C-2; and 129.8, C-6) indicated that the ethylamine group was connected to the 3-bromo-4-hydroxybenezene moiety. gHMBC correlations from H-7 (δ_H 3.00, 2.95) and H-8 (δ_H 4.44) to a carboxyl carbon (δ 173.2) suggested that the methine H-8 from the ethylamine group

Table 1 ^{1}H (600 M) and ^{13}C (150 M) NMR data for compounds **1-3** (DMSO-d_6)

Position	**1**			**2**			**3**		
	$\delta_H\delta_c$		HMBC	δ_H	δ_cHMBC		δ_H	δ_cHMBC	
1		130.1			133.4			129.4	
2	7.23 d (2.1)	133.7	C3, 4, 6, 7	8.03 s	133.6	C1, 3, 4, 7	7.28 d (2.2)	133.2	C1, 3, 4, 7
3		109.4			117.7			109.6	
4		153.3			152.4			152.9	
5	6.79 d (8.3)	116.6	C3, 4, 6		117.7		6.84 d(8.2)	116.7	C1, 3, 4
6	6.92 dd (8.3, 2.1)	129.8	C1, 4, 5	8.03 s	133.6	C1, 3, 4, 7	6.98 dd(2.2, 8.2)	129.4	C2, 4, 5, 7
7	3.00 dd (14.0, 5.0)	35.2	C1, 2, 8, 9	6.33 s	106.5	C2, 8, 9	2.56 t(7.3)	34.4	C1, 2, 6, 8
	2.95 dd (14.0, 8.2)								
8	4.44 m	54.1	C7, 9		143.8		3.18 t(7.3)	40.8	C1, 7, 1'
9		173.2			166.3				
1'		136.7						169.8	
2'	7.40 s	133.2	C3',6', 4', 7'				1.74 s	23.4	C1'
3'		117.6							
4'		152.6							
5'		117.6							
6'	7.40 s	133.2	C2',3', 4', 7'						
7'	3.73 d (14.2)	28.3	C1',2', 8', 9'						
	3.70 d (14.2)								
8'		151.1							
9'		163.3							
OCH_3	3.74 s	60.9	C4'	3.77 s	61.0				
OH	9.98 s		C3, 4, 5				9.94 s		
NH	7.87 d (7.9)		C8, 9'				7.82 t(5.6)		C1'
N-OH	12.11 s		C8'						

was connected to a carboxyl group. Thus a trisubstituted tyrosine moiety was assigned. In addition, a two-proton singlet (δ_H 7.40), characteristic of a symmetrical 1,3,4,5-tetrasubstituted benzene, showed gHMBC correlations to the quaternary aromatic carbons (δ_C 117.6, C-3', 5'; 152.6, C-4') and the methylene carbon (δ_C 28.3, C-7'), while the methylene (δ_H 3.73, H-7'a; 3.70, H-7'b) showed gHMBC correlations with the aromatic carbons (δ_C 136.7, C-1'; 133.2, C-2', 6'), and two quaternary carbons (δ_C 151.1, C-8'; 163.3, C-9'), indicating an α-oximebenzenepropanamide. The gHMBC correlation from the methoxy (δ_H 3.74, s) to the aromatic carbon (δ_C 152.6) suggested that methoxy group was attached to the C-4 position of 1,3,4,5-tetrasubstituted benzene. The configuration of the oxime was determined to be *E* by the diagnostic carbon chemical shift of the benzylicmethylenes (δ_C 28.3). The carbon chemical shift of benzylic methylene corresponding to Z oximines are known to be >35 ppm.[16]Finally theconnection of the α-oximebenzenepropanamide with the substituted tyrosine through an amide bond was established by thegHMBC correlations from the methine (δ_H 4.44, H-8) and the exchangeable NH proton (δ_H 7.87) to thecarbonyl carbon (δ_C 163.3)inoximebenzenepropanamide moiety. Thus, the planar structure of callyspongic acid was elucidated as **1**.

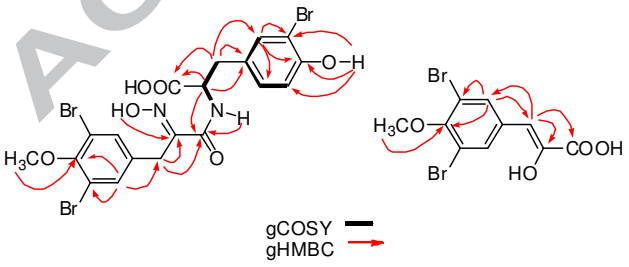


Figure 1: Key gCOSY and gHMBC correlations of **1** and **2**

The Marfey's reaction was not performed on compound **1**due to limited amount of compound obtained. However, the optical rotation of **1**($[\alpha]_D^{23}$ +3.63, *c* 0.008, MeOH) was compared with those of *L*-3-bromotyrosine ($[\alpha]_D^{23}$ -28, *c* 0.1, MeOH) and*D*-3-bromotyrosine ($[\alpha]_D^{23}$ +28, *c* 0.1, MeOH), indicating a *D*-3-bromotyrosine moiety in **1**. *D/L*-3-bromotyrosine was prepared from corresponding tyrosine (Fig. 2).

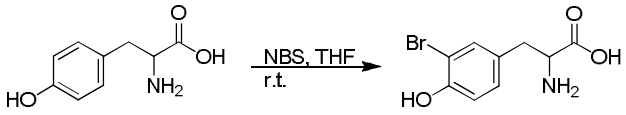


Figure 2: preparation of *D/L*-3-bromotyrosine

3,5-Dibromo-4-methoxyphenylpyruvic acid (**2**)[17]exhibited a cluster of isotopic ions at *m/z* 349/351/353 (1:2:1) in the (-)-LRESIMS spectrum, indicating the presence of two bromine atoms in the molecule. The molecular formula, $C_{10}H_8Br_2O_4$, was established on the basis of HRESIMS (*m/z* 348.8716 [M-H]$^-$, calcd348.8710 for $C_{10}H_7{}^{79}Br_2O_4$), with six degrees of unsaturation. The ^{1}H, and gHSQC NMR data of compound **2** showed a two-proton singlet (δ_H 7.79; δ_C 133.6), representing a symmetrical 1,3,4,5-tetrasubstituted benzene moiety, a methine singlet (δ_H 6.33; δ_C 106.5), and a methoxy (δ_H3.77; δ_C 61.0). The aromatic methine proton (δ_H 7.79) showed gHMBC correlations with the aromatic carbons (δ_C133.4, C-1; 117.7, C-3/5; and 106.5, C-7) and a sp^2 carbon (δ_C106.5, C-7). The methinesinglet (δ_H 6.33) showed gHMBC correlations with aromatic carbon (δ_C133.6, C-2/6), a sp^2 carbon (δ_C 143.8, C-8), and a carbonyl carbon (δ_C 166.3, C-9). ThesegHMBC correlation data indicated that compound **2** was very similar to 3-bromo-4-methoxyphenylpyruvic acid (enol form).[18, 19]Themethoxy group was located on the C-4 of the benzene ring by the gHMBC correlations from the methoxy proton (δ_H3.77) to the aromatic carbon (δ_C 152.4). Thus, the structure of **2** was assigned as 3,5-dibromo-4-methoxyphenylpyruvic acid.

N-acetyl-3-bromo-4-hydroxylphenylethamine (**3**)[20]was isolated as brown amorphous powder. (+)-LRESIMS showed an isotopic ion cluster at *m/z* 258/260 (1:1), indicating the presence of one bromine atom in the molecule. The molecular formula of **3**was determined to be $C_{10}H_{12}BrNO_2$, on the basis of HRESIMS (*m/z* 279.9943 $[M+Na]^+$, calcd279.9940 for $C_{10}H_{12}{}^{79}BrNO_2Na$). The ^1H, and gHSQC NMR data of compound **3**showed two exchangeable protons (δ_H9.94 s; 7.82, t, J = 5.6 Hz), three mutually coupled aromatic protons (δ_H 7.28, d, J = 2.2 Hz, H-2; 6.98, dd, J = 2.2, 8.2 Hz, H-6; and 6.84, d, J = 8.2 Hz, H-5), two methylenes (δ_H3.18, t, J = 7.3 Hz; 2.56, t, J = 7.3 Hz), and a methyl group (δ_H1.74 s). The above data were very similar to those of *N*-acetyl-3,5-dibromo-4-hydroxyl phenylethamine (**4**). The only difference was that compound **3** had an 1,3,4-trisubstituted benzene ring instead of a symmetrical 1,3,4,5-tetrasubstituted benzene ring in **4**. Hence,compound**3** was assigned as *N*-acetyl-3-bromo-4-hydroxylphenylethamine.

Ten known natural products, namely *N*-acetyl-3,5-dibromo-4-hydroxylphenylethamine (**4**), 3,5-dibromo-4-methoxyphenylacetic acid(**5**),comantherin (**6**), bastadin 6 (**7**), bastadin 7 (**8**),bastadin 8 (**9**),bastadin 9 (**10**),bastadin 16 (**11**),bastadin 18 (**12**),andbastadin 24 (**13**), were also isolated from the marine sponge. Their structures were identified by comparing their ^1H and ^{13}C NMR data with those reported in the literature. Interesting, 3,5-dibromo-4-methoxyphenylpyruvic acid (**2**) was the rare example of natural occurring phenylpyruvic acid (enol) derivatives. This is also the first report of the presence of bastadins from the sponge *Callyspongia* sp.

The ApoE modulatory activities of compounds **1**-**13** were evaluated. 3,5-Dibromo-4-methoxyphenylpyruvic acid (**2**)was the only active compound, increasingApoE secretion by one fold over background at the concentration of 40 μM.

In conclusion, thirteen compounds (**1**-**13**) were isolated from the Australian marine sponge *Callyspongia* sp. Three compounds (**1**-**3**) are new to science and 3,5-dibromo-4-methoxyphenyl-pyruvic acid (**2**) show weak ApoE modulation activities. It is the first time that bastadins were isolated from the sponge *Callyspongia* sp.

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Supplementary data

Supplementary data (^1H, gCOSY, gHSQC, gHMBC NMR spectra for compounds **1**-**3**,general experimental procedures, spongecollection and identification, extraction and isolation procedures, Preparation of *D*-and *L*-3-bromotyrosine, ApoE modulation activity assay) associated with this article can be found, in the online version, at

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15. Compound **1**, isolated as brown amorphous powder; $[\alpha]_D$ +3.63 (c 0.008, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 282 (3.75), 211 (4.68) nm; IR (KBr) ν_{max} 3380, 2924, 2854, 1712, 1497, 1260, 1203 cm^{-1};^1H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 628.8529 $[M+Na]^+$ (calcd for $C_{19}H_{17}{}^{79}Br_3N_2O_6Na$, 628.8532).
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17. Compound **2**, isolated as pale amorphous powder; UV (CH_3OH) λ_{max} (log ε) 292 (3.54), 209 (4.39) nm; IR (KBr) ν_{max} 3368, 2930, 2582, 1764, 1706, 1267, 997 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 348.8716 $[M-H]^-$ (calcd for $C_{10}H_7{}^{79}Br_2O_4$, 348.8711).
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20. Compound **3**, isolated as pale amorphous powder; UV (CH_3OH) λ_{max} (log ε) 283 (3.34), 210 (4.07) nm; IR (KBr) ν_{max} 3274, 2927, 1647, 1508, 1288, 1197 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 279.9943 $[M+Na]^+$ (calcd for $C_{10}H_{12}{}^{79}BrNO_2Na$, 279.9940).