ISOLATION AND STRUCTURAL ELUCIDATION OF BIOACTIVE C-12, 15-*CIS* NON-ADJACENT BIS-THF ANNONACEOUS ACETOGENINS

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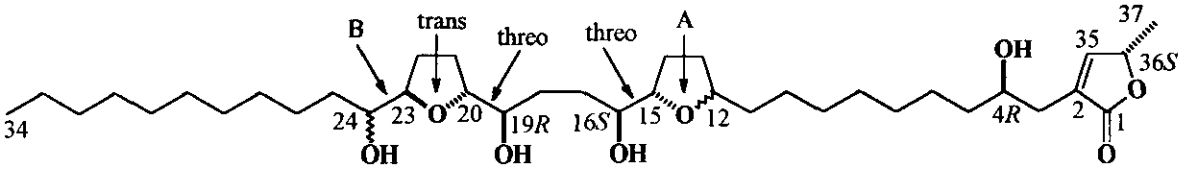
Abstract- In further investigation on the EtOH extract of the bark of *Annona bullata*, two novel Annonaceous acetogenins, C-12,15-*cis*-bullatanocin (**1**) and C-12,15-*cis*-bullatalicin (**2**), were successfully separated by hplc from fractions in which they were mixed with bullatanocin (**7**) and bullatalicin (**8**), their respective C-12/15-*trans* diastereomers. Their relative stereochemistries between the two THF rings were determined through preparations of their respective 16,19-formaldehyde acetal derivatives (**1a** and **2a**). The absolute configurations of **1** and **2** were resolved by analyses of the ^{1}H nmr data of the (*S*)- and (*R*)-Mosher ester derivatives (**1b**, **1c** and **2b**, **2c**) of **1a** and **2a**. Compounds (**1**) and (**2**) are the first examples of C-12/15-*cis* non-adjacent bis-THF Annonaceous acetogenins and showed potent bioactivities, comparable to those of **7** and **8**, with notable cytotoxic selectivities among six human solid tumor cell lines.



	A	B		A	B
1	*cis* (12*S*)	*threo* (24*R*)	**7**	*trans* (12*R*)	*threo* (24*R*)
2	*cis* (12*S*)	*erythro* (24*S*)	**8**	*trans* (12*R*)	*erythro* (24*S*)

So far, over 160 Annonaceous acetogenins have been reported, and most of them show various types of potent bioactivities which, at least in part, are due to mitochondrial inhibition.[1] Many of these compounds are diastereomers and differ from each other only in the configurations at one or two stereogenic centers. For

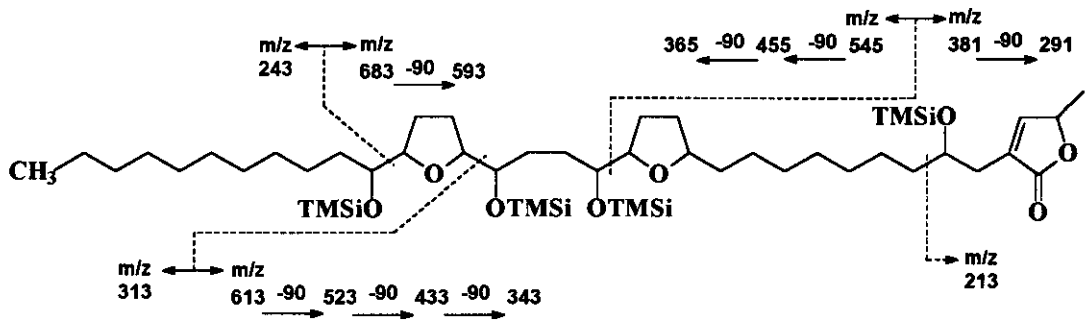


Figure 1. Diagnostic EIms fragment ions of the TMSi derivatives of **1**, **2**, **7**, and **8**.

example, the difference between bullatanocin (**7**) and bullatalicin (**8**) lies in the absolute configuration at C-24, which is *R* in **7** and *S* in **8**.[1b] Because of their close chemical similarities, purification of such compounds, except by hplc, is often difficult. When we checked certain fractions of the EtOH extract of the bark of *Annona bullata* Rich., that were known to contain **7** or **8**, ^{1}H nmr signals at δ 3.72, with low intensities, indicated that possible isomers exist in low quantity. Repeated purification of such fractions by normal phase hplc with a silica gel column did not improve the purity. Finally, two new isomeric compounds, (**1**)[2] and (**2**)[3], were successfully separated from **7** and **8**, respectively, by reversed phase hplc using a C_{18} column.

EIms fragmentations (Figure 1) of the TMSi derivatives of **1**, **2**, **7**, and **8** were indistinguishable from each other and demonstrated that all of the four compounds possess the same carbon skeleton. The ^{1}H and ^{13}C nmr data (Figure 2, Tables 1 and 2) of the four compounds suggested that they have different configurations in the non-adjacent bis-THF subunits, which are clearly exhibited in their partial ^{1}H nmr spectra (Figure 2).

All of the ^{1}H and ^{13}C nmr data of **1** and **7** are very similar except for those representing the non-adjacent bis-THF subunits. In the ^{1}H nmr spectra of **1** and **7**, three proton signals, appearing at *ca*. δ 3.4, for H-16, 19,

Table 1. ^{13}C Nmr (125 MHz, $CDCl_3$) Data of the C-12/15 THF Subunit of **1-12** and Model Compounds.

threo-cis							*threo-trans*						
C-No.	11	12	13	14	15	16	C-No.	11	12	13	14	15	16
mod[a]	36.1	79.9	31.4	27.8	82.2	74.5	mod[a]	35.7	79.3	32.4	28.4	81.9	74.2
1	35.9	79.8	31.2	27.7	82.3	74.7	**7**	35.5	79.3	32.3	28.4	82.0	74.4
2	36.0	79.9	31.4	27.8	82.3	74.8	**8**	35.6	79.3	32.4	28.4	82.0	74.5
3 & 4	35.9	79.8	31.2	27.7	82.3	74.7	**9 & 10**	35.5	79.3	32.3	28.4	82.0	74.3
5 & 6	35.9	79.8	31.4	27.7	82.2	74.8	**11 & 12**	35.6	79.2	32.4	28 4	82.0	74.5

a: Data of model compounds taken from Fujimoto *et al*.[4a]

Table 2. ^{13}C Nmr (125 MHz, $CDCl_3$) Data of the C-19/26 THF Subunit of **1-6** and Model Compounds.

C-No.	19	20	21	22	23	24	25	26
t-t-t[a]	74.0	82.7	28.8	28.8	82.7	74.0	33.4	25.5
1	74.3	82.7	28.7	28.7	82.7	74.1	33.5	25.6
2 & 3	74.3	82.7	28.7	28.7	82.7	74.1	33.3	25.6
t-t-e[b]	74.3	83.3	28.6	25.2	82.2	71.6	32.5	25.9
4	74.5	83.3	28.6	25.2	82.2	71.5	32.5	26.0
5 & 6	74.5	83.3	28.6	25.2	82.2	71.4	32.5	26.0

a,b: Data of model compounds from Fujimoto *et al.*;[4a] a: *threo-trans-threo*; b: *threo-trans-erythro*.

and 24, indicated that the relative stereochemistries of C-15/16, C-19/20, and C-23/24 are all *threo*.[4] One of the methylene protons of C-21 and 22, resonating at *ca.* δ 2.00, and the other one, resonating at *ca.* δ 1.60, suggested that the relative configurations of the C-20/23 THF ring of **1** and **7** are *trans*.[4a,5] Therefore, the difference between **1** and **7** must be within the configuration of the C-12/15 THF ring. The H-15 resonated at δ 3.80 in **7** and was shifted upfield to δ 3.72 in **1**. This assignment was confirmed by single- and double-relayed COSY spectra. The unusual chemical shift of H-15 reminded us of that of muricatetrocin B, which also possesses a THF ring at C-12/15 with a single flanking hydroxyl.[6] The H-15 of muricatetrocin-B resonates at δ 3.72 and the configuration of its THF ring was determined to be *cis*.[6] Thus, the configuration of the C-12/15 THF ring in **1** was also suggested to be *cis* and not *trans* as in **7**. This conclusion was further demonstrated to be correct by the NOESY spectrum of **1**, in which the correlation cross peaks between H-15 (at δ 3.72) and H-12 (at δ 3.86) can be clearly observed. Thus, the relative stereochemistry of the non-adjacent bis-THF subunit (C-12 to C-24) of **1** was determined to be *cis-threo-threo-trans-threo*.

As we were preparing this manuscript, a very helpful paper from Fujimoto's group appeared in the literature.[4a] They synthesized a series of model mono-THF ring compounds with all the possible relative stereochemistries

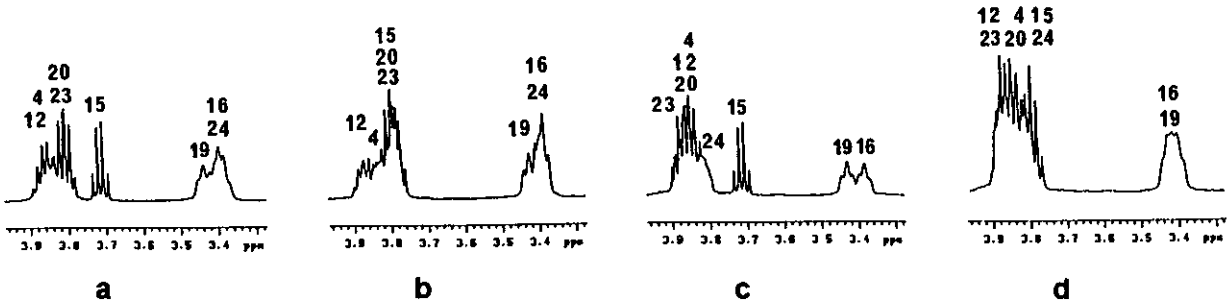
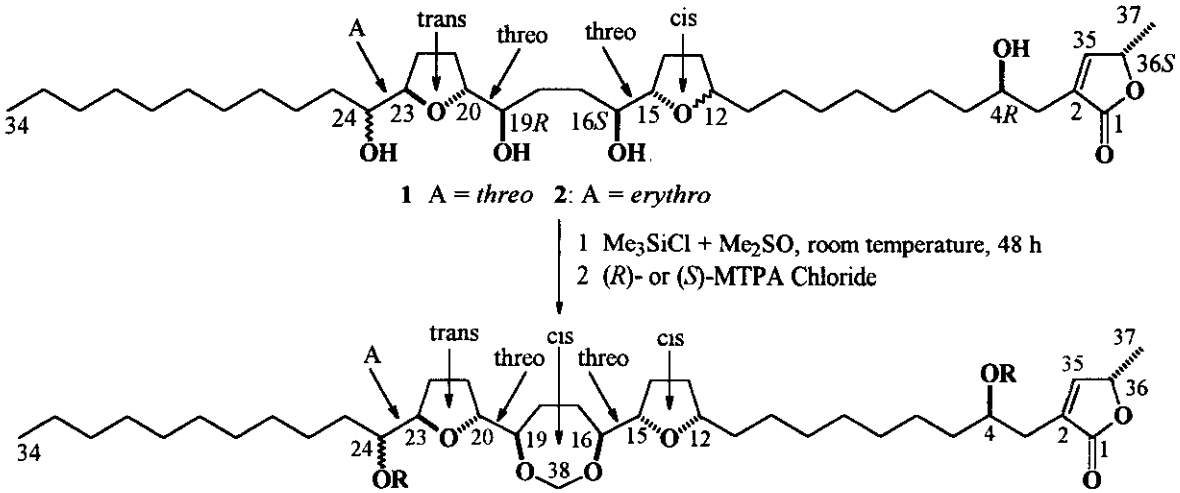


Figure 2. Parital ^1H Nmr (500 MHz, $CDCl_3$) Spectra of **1** (a), **2** (c), **7** (b), and **8** (d).

and reported the ^1H and ^{13}C resonances. The ^1H (Figure 2) and ^{13}C (Tables 1 and 2) nmr data of C-10 to C-16 of **1** match well with those of the model single-hydroxyl flanked THF ring with a *cis-threo* relative configuration (Table 1), while those of C-19 to C-26 agree well with those of the model mono-THF ring having a flanking hydroxyl on both sides and a *threo-trans-threo* configuration (Table 2). These comparisons further demonstrate that the proposed structure of **1** is correct.



	1a	1b	1c	2a	2b	2c
A	*threo*	*threo*	*threo*	*erythro*	*erythro*	*erythro*
R	H	R-MTPA	S-MTPA	H	R-MTPA	S-MTPA

The absolute configuration of **1** was determined through the preparation of the formaldehyde acetal derivative (**1a**)[7] of **1** and the *S*- and *R*-Mosher ester [methoxy(trifluoromethyl)phenyl acetate or MTPA] derivatives (**1b** and **1c**)[8] of **1a**. In the ^1H nmr spectrum of **1a**, the acetal proton signals appeared as two well-separated doublets at δ 5.258 and 4.636. This indicated that the configuration of the acetal ring of **1a** is *cis*, and, thus, all of the relative stereochemical centers of **1**, among the two mono-THF subunits and between them, are defined. The analyses of the differences of the proton chemical shifts of **1b** and **1c** suggested the *R* absolute configuration at C-4, as is usual, and, also, the *R* configuration at C-24.[8] The structure of **1** was, thus, established as illustrated with the defined absolute configuration of 4*R*, 12*S*, 15*S*, 16*S*, 19*R*, 20*R*, 23*R*, 24*R*, and 36*S*. Compound (**1**) was named C-12,15-*cis*-bullatanocin.

Compounds (**2**) and (**8**) differ from **1** and **7** only in the relative stereochemistries of the THF-flanking hydroxylated carbon centers and the THF rings. In the ^1H nmr spectra of **2** and **8**, only two signals, not three as in those of **1** and **7**, appeared at *ca.* δ 3.40, corresponding to three *threo* relative configurations.[4] One of

the protons at H-16, 19, and 24 of **2** and **8** resonated at δ 3.88 (Figure 2), corresponding to a relative configuration of *erythro*.[4] The assignment of the *erythro* configuration in **2** at C-23/24 was aided by the preparation of the 16,19-formaldehyde acetal derivative (**2a**) of **2**. In the ^{1}H nmr spectrum of **2a**, there were no signals at *ca.* δ 3.40; both proton signals at *ca.* δ 3.40 in **2** were shifted downfield to *ca.* δ 3.60 in **2a**. Therefore, the two proton signals of **2** at *ca.* δ 3.40 could be assigned for H-16 and 19, and the relative configurations at C-15/16 and C-19/20 were determined to be *threo*. The H-24 in **2a** resonated at δ 3.88 and indicated that the relative configuration of C-23/24 is *erythro*.[4]

As in **1** and **7**, the difference between **2** and **8** was also concluded to be the configuration of the C-12/15 THF ring. The H-15 of **2** resonated at δ 3.72 and has cross peaks with H-12 at δ 3.86 in the NOESY spectrum. This indicates that the relative stereochemistry of the C-12/15 THF ring is *cis* in **2**, and not *trans* as in **8**. The good agreement of the ^{13}C nmr data of the signals for C-11 to C-16 of **2** (Table 1), with those of the model single-hydroxyl flanked mono-THF compound of Fujimoto *et al.*[4a] having a *cis-threo* relative stereochemistry, confirmed this conclusion. The acetal proton signals in **2a**, appearing as doublets at δ 5.293 and 4.643 (Table 3), defined the *R/S* or *S/R* relative stereochemical relationship between C-16 and C-19 of **2**.[7] The absolute configurations of C-4 and C-24 were determined to be *R* and *S*, respectively, by analyses of the ^{1}H nmr chemical shifts of the (*S*)- and (*R*)-Mosher ester derivatives (**2b** and **2c**) of **2a**.[8] Consequently, the absolute configuration of **2** was deduced to be 4*R*, 12*S*, 15*S*, 16*S*, 19*R*, 20*R*, 23*R*, 24*S*, and 36*S*. Thus, the structure of **2** was established as illustrated and named C-12,15-*cis*-bullatalicin.

Table 3. ^{1}H Nmr (500 MHz, $CDCl_3$) Data of C-11 to C-19 of **1a** and **2a**.

H-No.	11	12	13a	13b	14a	14b	15	16	17 & 18	19
1a	1.52	3.84	1.94	1.47	1.86	1.62	3.90	3.67	1.86	3.66
2a	1.52	3.84	1.94	1.47	1.84	1.63	3.89	3.63	1.84	3.66
H-No.	20	21a	21b	22a	22b	23	24	25	38a	38b
1a	4.00	1.96	1.70	1.97	1.62	3.84	3.39	1.38	5.258	4.635
2a	4.04	2.01	1.64	1.90	1.84	3.96	3.88	1.35	5.294	4.642

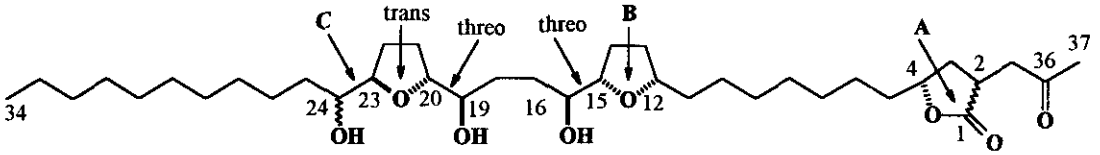
Compounds (**1**) and (**2**) are the first examples of non-adjacent bis-THF acetogenins having a *cis* configuration in the C-12/15 THF ring.[1] The differences between **1** and **2** vs. **7** and **8** are the configurations at C-12, *i.e.*, *S* in **1** and **2** and *R* in **7** and **8**. The differences between **1** and **7** vs. **2** and **8** are the configurations at C-24, *i.e.*, *R* in **1** and **7** and *S* in **2** and **8**. A biogenetic hypothesis for the formation of the non-adjacent bis-THF rings is

Table 4. ^{1}H Nmr (500 MHz, $CDCl_3$)Data of the (*S*)- and (*R*)-Mosher Esters of **1** (**1b** and **1c**) and **2** (**2b** and **2c**).

H-No.	1b	1c	$\Delta\delta_H$ (δ_S - δ_R)	2b	2c	$\Delta\delta_H$ (δ_S - δ_R)
37	1.29	1.32	- 0.03	1.29	1.32	- 0.03
36	4.86	4.91	- 0.05	4.86	4.91	- 0.05
35	6.72	6.98	- 0.26	6.72	6.98	- 0.26
3a	2.60	2.68	-0.08	2.60	2.68	-0.08
3b	2.57	2.61	- 0.04	2.57	2.61	- 0.04
4	5.31	5.37	***R***[a]	5.31	5.37	***R***[a]
5	1.65	1.62	+ 0.03	1.64	1.62	+ 0.02
20	3.93	4.01	- 0.08	3.93	3.72	+ 0.21
21a	1.75	1.92	- 0.17	1.90	1.81	+ 0.09
21b	1.62	1.75	- 0.13	1.68	1.62	+ 0.06
22a	1.90	2.01	- 0.11	1.93	1.85	+ 0.08
22b	1.50	1.57	- 0.07	1.78	1.70	+ 0.08
23	4.08	4.08	0	4.13	4.05	+ 0.08
24	5.06	5.05	***R***[a]	5.33	5.29	***S***[a]
25	1.62	1.48	+ 0.14	1.60	1.62	- 0.02

a: Absolute configuration of carbinol center.

proposed in Figure 3; this scheme can explain the possible biogenetic pathways leading to the four compounds. The *erythro* configuration of C-23/24 in **2** and **8** is possibly derived from the *trans* C-23/24 double bond in the proposed precursors. The *cis* and *trans* configurations of the C-12/15 THF rings are derived from the *S* or *R* configurations of C-12 caused by the reduction of the C-12 ketones in the proposed precursors.



	A	B	C		A	B	C
3	*cis* (2*R*)	*cis* (12*S*)	*threo* (24*R*)	**9**	*cis* (2*R*)	*trans* (12*R*)	*threo* (24*R*)
4	*trans* (2*S*)	*cis* (12*S*)	*threo* (24*R*)	**10**	*trans* (2*S*)	*trans* (12*R*)	*threo* (24*R*)
5	*cis* (2*R*)	*cis* (12*S*)	*erythro* (24*S*)	**11**	*cis* (2*R*)	*trans* (12*R*)	*erythro* (24*S*)
6	*trans* (2*S*)	*cis* (12*S*)	*erythro* (24*S*)	**12**	*trans* (2*S*)	*trans* (12*R*)	*erythro* (24*S*)

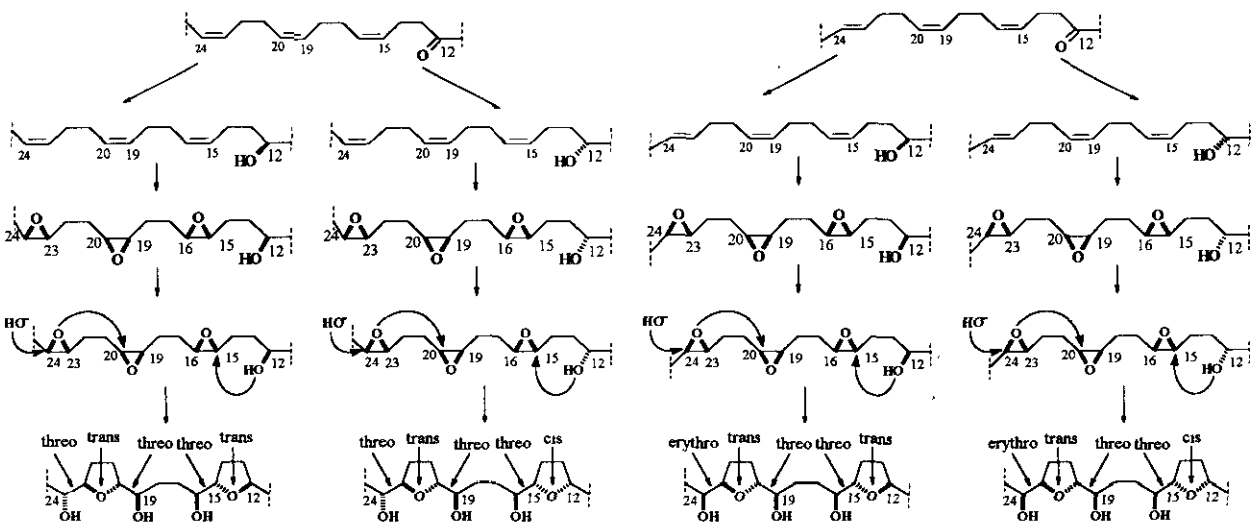
The absolute resolution of the structures of **1** and **2** led us to re-examine the ^{1}H and ^{13}C nmr spectra of some chromatographic fractions from *A. bullata* containing (2,4-*cis* and *trans*)-bullatanocinones (**9** and **10**) or (2,4-*cis* and *trans*)-bullatalicinones (**11** and **12**).[1] The results showed that similar low intensity ^{1}H nmr peaks at

Table 5 . Bioactivities of Compounds **1** - **8**.[a]

	BSTb LC_{50}(μg/ml)	A-549[c] ED_{50}(μg/ml)	MCF-7[d] ED_{50}(μg/ml)	HT-29[e] ED_{50}(μg/ml)	A-498[f] ED_{50}(μg/ml)	PC-3g ED_{50}(μg/ml)	MIA PaCa-2[h] ED_{50}(μg/ml)
1	5.60×10^{-1}	1.51×10^{-8}	8.53×10^{-4}	5.81×10^{-3}	8.23×10^{-3}	6.81×10^{-4}	1.25×10^{-15}
2	8.19×10^{-1}	1.88×10^{-11}	> 1.0	> 1.0	3.48×10^{-5}	2.15×10^{-6}	2.81×10^{-14}
7	4.33×10^{-1}	2.15×10^{-11}	2.10×10^{-1}	3.67×10^{-6}	6.39×10^{-3}	4.39×10^{-2}	8.90×10^{-15}
8	1.24	1.16×10^{-13}	2.79×10^{-1}	> 1.0	2.59×10^{-2}	1.99×10^{-7}	6.09×10^{-15}
Adr.[i]	8.00×10^{-2}	3.87×10^{-3}	7.23×10^{-1}	1.98×10^{-2}	2.66×10^{-2}	8.76×10^{-2}	3.14×10^{-2}

a) To permit optimal comparisons, all of the samples were tested in the same run in each bioassay; b) Brine shrimp lethality test;[9a] c) Human lung carcinoma;[9b] d) Human breast carcinoma;[9c] e) Human colon adenocarcinoma;[9d] f) Human kidney carcinoma;[9b] g) Human prostate adenocarcinoma;[9e] h) Human pancreatic carcinoma;[9f] i) Adriamycin--Standard positive control.

δ3.72 also appeared in these fractions, indicating the presence of the new ketolactone compounds (**3-6**) having *cis* configurations of their C-12/15 THF rings. The partial ^{13}C nmr resonances for the non-adjacent bis-THF subunit of **3-6** and **9-12** are compared in Tables 1 and 2. These data, being very similar to those of their respective 4-OH, α,β-unsaturated γ-lactone acetogenins (**1**, **2**), support the illustrated structures of **3-6**. Thus, **3** and **4** are named (2,4-*cis* and *trans*)-C-12,15-*cis*-bullatanocinones and **5** and **6** are named (2,4-*cis* and *trans*)-C-12,15-*cis*-bullatalicinones, respectively.



Bullatanocin (**7**) 12,15-*cis*-Bullatanocin (**1**) Bullatalicin (**8**) 12,15-*cis*-Bullatalicin (**2**)

Figure 3. Hypothesis for the biogenesis of the non-adjacent bis-THF rings **1**, **2**, **7**, and **8**.

Like **7** and **8**, **1** and **2** also showed very good bioactivities in the brine shrimp lethality test (BST) and potent cytotoxicities among six solid human tumor cell lines (Table 5).[9] For example, all of the compounds (**1**, **2**,

7, and **8**) are potently effective and selective against the A-549 (lung) and the MIA PaCa-2 (pancreatic) cell lines (Table 5). Bullatalicin (**8**), the non-adjacent bis-THF acetogenin with a C-12/15 *trans*-THF ring, has previously shown very good efficacy, comparable to that of cisplatin,[10] against human A2780 ovarian tumor xenografts in athymic mice. The C-12/15 *cis*-THF non-adjacent bis-THF acetogenins now provide additional choices in the investigation of optimal structure-activity relationships and in the discovery of the most efficaceous acetogenins for antitumor drug development.

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