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TWO NEW CYTOTOXIC MONOTETRAHYDROFURAN ANNONACEOUS ACETOGENINS, ANNOMURICINS A AND B, FROM THE LEAVES OF *ANNONA MURICATA*

FENG-E WU, ZHE-MING GU, LU ZENG, GENG-XIAN ZHAO,
YAN ZHANG, JERRY L. MCLAUGHLIN,*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907

and SOELAKSONO SASTRODIHARDJO

Department of Education and Culture, Center of Interuniversities, Biology Division, Bandung Institute of Technology, 10 Yanesba St., Bandung 40132, Indonesia

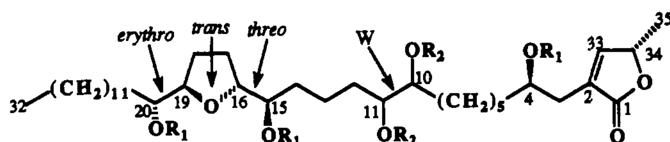
ABSTRACT.—The leaves of *Annona muricata* have yielded eight monotetrahydrofuran Annonaceous acetogenins. Two of them, annomuricins A [**1**] and B [**2**], whose chemical structures were deduced by ms, nmr, ir, and uv spectral and chemical methods, are novel and unusual. Compounds **1** and **2** each possess five hydroxyl groups; two hydroxyl groups are vicinal, with the vicinal group of **1** threo and that of **2** erythro. The absolute configurations of **1** and **2** were determined by Mosher ester methodology. Six monotetrahydrofuran acetogenins, previously described in the seeds, were found in the leaves; these are gigantetrocin A, annonacin-10-one, muricatetrocins A and B, annonacin, and goniothalamicin.

Annona muricata L. (Annonaceae) is a small tropical tree whose edible fruits are used commercially for the production of juice, candy, and sherbets. The seeds, as a byproduct of this industry, are an abundant biomass source of the Annonaceous acetogenins. Previous studies on the seeds have resulted in the bioactivity-directed isolation and characterization of a number of cytotoxic and pesticidal monotetrahydrofuran acetogenins as well as their precursors or metabolites (1–9). In the present work, the leaves of *Annona muricata* have yielded two new Annonaceous acetogenins, named annomuricins A [**1**] and B [**2**]. Six known monotetrahydrofuran acetogenins, gigantetrocin A, annonacin-10-one, muricatetrocins A and B, annonacin, and goniothalamicin, were also isolated from the leaves; these have been previously found in the seeds (3–6).

RESULTS AND DISCUSSION

The dried powdered leaves of *A. muricata*, obtained from plantation trees growing in Java, were extracted with 95% EtOH; the residue of the extract (F001) was partitioned through a standard extraction scheme (see Experimental), and the partitions were evaluated for toxicity with a test for lethality to brine shrimp larvae (BST) (10,11). The most active fraction (F005, BST LC_{50} 0.17 μg/ml) was subjected to flash chromatography over Si gel eluted by gradients of hexane/EtOAc and EtOAc/MeOH. The fractions were analyzed by tlc and evaluated in the BST. An active fraction (No. 128) was further subjected to repeated flash chromatography and hplc to yield compounds **1** and **2**. The known compounds were isolated from active fractions (Nos. 86–127) using similar methods.

Annomuricins A [**1**] and B [**2**] were obtained as colorless amorphous powders. The hrfabms gave $[MH]^+$ ions at *m/z* 613.4691 [**1**] and 613.4694 [**2**] (calcd 613.4679), both consistent with the molecular formula of $C_{35}H_{64}O_8$. The ms and nmr spectra indicated that **1** and **2** are mono-THF ring acetogenins (4–6) (Figure 1). Both **1** and **2** showed a broad OH stretching absorption in their ir spectra at 3250–3550 cm^{-1}. Five successive losses of H_2O (*m/z* 18) from the $[MH]^+$ from **1** and **2** in the cims (*m/z* 595, 577, 559, 541, and 523) demonstrated the existence of five OH groups, and these were confirmed by the

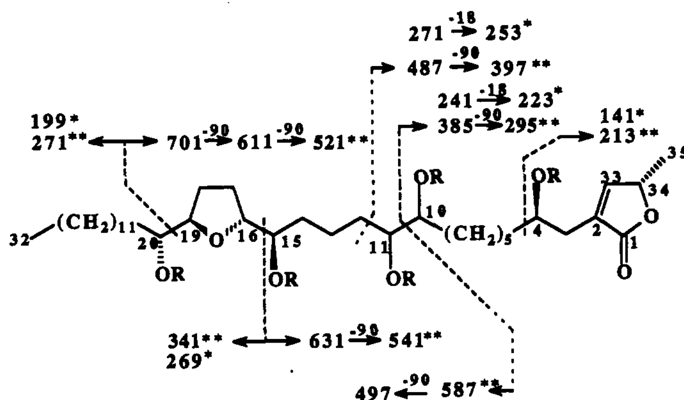


Compound	W	R_1	R_2
1	threo	H	H
1a	threo	Ac	Ac
1b	threo	TMSi	TMSi
1c	threo	H	Acetonide
1s	threo	(*S*)-MTPA	(*S*)-MTPA
1r	threo	(*R*)-MTPA	(*R*)-MTPA
2	erythro	H	H
2a	erythro	Ac	Ac
2b	erythro	TMSi	TMSi
2c	erythro	H	Acetonide
2s	erythro	(*S*)-MTPA	(*S*)-MTPA
2r	erythro	(*R*)-MTPA	(*R*)-MTPA

FIGURE 1. Structures of annomuricins A [**1**], B [**2**], and their derivatives (**1a–c**, **1s**, **1r**, **2a–c**, **2s**, **2r**).

formation of pentaacetates (**1a** and **2a**) and penta-trimethylsilyl (TMSi) ethers (**1b** and **2b**). Pentaacetates **1a** and **2a** gave five singlet proton peaks at δ 2.02–2.08 (Table 1). The positions of the OH groups in **1** and **2** were assigned at C-4, C-10, C-11, C-15, and C-20 by careful analysis of the fragments in the eims spectra of the TMSi derivatives (**1b** and **2b**) at *m/z* 701, 631, 487, 385, 341, 271, and 213 (Figure 2). The hrcims of both **1** and **2** gave a $[C_{19}H_{37}O_4Si_2]^+$ ion at *m/z* 385.2239 (calcd 385.2230), which confirmed the presence of a vicinal diol at C-10/C-11. The placement of the mono-THF ring was determined to be at C-16 by the diagnostic fragments at *m/z* 631 and 341 (TMSi-eims).

The mono-THF ring, with the usual OH groups on each side, was indicated in **1** and **2** by ^{1}H-nmr chemical shifts (Table 1) at δ 3.41 (H-15) and 3.80–3.89 (H-16, H-19, and H-20) and the ^{13}C-nmr signals (Table 1) at δ 74.39 [**1**] and 74.53 [**2**] (C-15), 83.30 (C-16), 82.27 (C-19), and 71.53 (C-20); however, the configurations of the vicinal diol at



* R=H
** $R=Me_3Si$

FIGURE 2. Diagnostic eims fragment ions (*m/z*) of **1** and **2** (R=H) and their Tri-TMSi derivatives (**1b** and **2b**, $R=Me_3Si$).

TABLE 1. [1]H-Nmr Spectral Data for **1**, **2**, **1a**, **2a**, **1c**, and **2c**, and ^{13}C-Nmr Data for **1** and **2** ($CDCl_3$, δ).

Position	[1]H Nmr (500 MHz)						[13]C Nmr	
	1	**2**	**1a**	**2a**	**1c**	**2c**	**1**	**2**
1	—	—					174.65	174.65
2	—	—					131.12	131.12
3a	2.37 m	2.38 m	2.51 m	2.51 m	2.37 m	2.38 m	33.35	33.34
3b	2.50 m	2.51 m	2.58 m	2.58 m	2.50 m	2.51 m		
4	3.81 m	3.81 m	5.10 m	5.09 m	3.82 m	3.80 m	69.82	69.82
5–9	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	22–34	22–34
10	3.43 m	3.61 m	4.97 m	4.93 m[a]	3.59 m	4.02 m[a]	74.15	74.23
11	3.43 m	3.61 m	4.97 m	4.96 m[b]	3.59 m	4.04 m[b]	74.27	74.35
12–14	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	23–34	22–34
15	3.41 m	3.41 m	4.85 m	4.85 m	3.41 m	3.46 m	74.39	74.53
16	3.85 m	3.85 m	3.95 m	3.95 m	3.86 m	3.88 m	83.30	83.30
17–18	1.57-2.00 m	1.57-2.00 m	1.57-2.00 m	1.57-2.00 m	1.57-2.00 m	1.57-2.00 m	22–34	22–34
19	3.80 m	3.80 m	3.95 m	3.95 m	3.80 m	3.80 m	82.27	82.27
20	3.89 m	3.89 m	4.92 m	4.92 m	3.90 m	3.90 m	71.53	71.53
21–31	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	1.25-1.61 m	22–34	22–34
32	0.88 t	0.88 t	0.88 t	0.88 t	0.88 t	0.88 t	14.10	14.10
33	7.19 d	7.19 d	7.09 d	7.09 d	7.09 d	7.09 d	151.90	151.90
34	5.05 dq	5.05 dq	5.01 dq	5.01 dq	5.06 dq	5.06 dq	78.01	78.01
35	1.42 d	1.43 d	1.39 d	1.39 d	1.43 d	1.42 d	19.08	19.08
4-OAc			2.03 s	2.02 s				
10-OAc			2.08 s	2.05 s				
11-OAc			2.08 s	2.05 s				
15-OAc			2.08 s	2.07 s				
20-OAc			2.05 s	2.03 s				

[a,b]Assignments are interchangeable.

C-10/C-11 showed differences between **1** and **2**. For **1**, the carbinol protons at C-10/C-11 resonated at δ 3.42 and 3.43, and, for **2**, they resonated at δ 3.60 and 3.61.

These structural units were further confirmed by COSY and single-relayed COSY data in which the proton coupling correlations from H-3↔H-4 and (H-10 and H-11) ↔ (H-8, H-9, H-12, and H-13) and (H-15 and H-20) ↔ (H-13, H-14, H-16, H-17, H-18, H-19, H-21, and H-22) (Table 1) could be clearly seen. The assignment of the relative stereochemistry around the mono-THF ring of **1** and **2** was determined using the methodology of Hoye and co-workers (12,13) and Born *et al.* (14), as well as by comparison with annonacin A (15), (2,4-*cis* and *trans*)-annonacin-A-one (16), and jetein (17) (Table 2). The THF ring location, at C-15 to C-20, of **1** and **2** is the same as in the above known compounds.

In **1** and **2**, the OH-substituted CH centers flanking the ring region (C-16 to C-19) gave identical chemical shifts in the [1]H- and ^{13}C-nmr spectra (Table 1); the stereochemistry of C-15/C-16 and C-19/C-20 was concluded to be threo and erythro, respectively, and the stereochemistry was trans for the THF ring (12,13). The relative stereochemistry around the mono-THF ring was confirmed by comparing the [1]H-nmr of the acetates (**1a** and **2a**, Table 1) with those of model compounds of known relative stereochemistry (12,14). The proton signals for H-15 at δ 3.41 and H-20 at δ 3.80 in **1** and **2**, were shifted downfield in **1a** and **2a** to δ 4.85 for H-15 and δ 4.92 for H-20.

TABLE 2. ^{1}H-Nmr (500 MHz) Signals for the Protons of the Threo and Erythro Diols in **1**, **2**, **1a**, **2a**, **1c**, and **2c**.

Configuration/ Compound	Methine Protons		Acetyl Methyls		Acetonyl Methyls	
	threo **1**	erythro **2**	threo **1a**	erythro **2a**	threo **1c**	erythro **2c**
Diol	3.43 (2H)	3.61 (2H)	—	—	—	—
Acetate	4.97 (2H)	4.96 (1H) 4.93 (1H)	2.08 (6H)	2.05 (6H)		
Acetonide	3.59 (2H)	4.02 (1H) 4.04 (1H)	—	—	1.37 (6H)	1.43 (3H) 1.33 (3H)

To determine the relative configuration at C-10/C-11, the acetonide (dioxolane) derivatives of **1** and **2** (**1c** and **2c**) were prepared (Table 2). The ^{1}H-nmr signals for H-10 and H-11 of threo vicinal diols have been previously reported in other Annonaceous acetogenins (18–20). Comparison with the spectral data of these compounds revealed the configuration of the diol in **1** to be threo. The ^{1}H-nmr signals for H-10 and H-11 in **2c**, at δ 4.02 and 4.04, and for the acetonyl methyls, showing two separate singlet peaks at δ 1.43 and 1.33, respectively, suggested a cis configuration for the dioxolane ring of **2c**. Thus, the configuration of the diol of **2** was determined to be erythro, since the cis configuration at C-10/C-11 could only be derived from a vicinal diol with an erythro configuration. Only one acetogenin with an erythro vicinal diol has been previously reported among the Annonaceous acetogenins (20).

Rieser *et al.* have reported the determination of the absolute configuration of stereogenic carbinol centers in several Annonaceous acetogenins using Mosher ester methodology (6,21). Thus, the (*S*)- and (*R*)-methoxyfluoromethyl-phenylacetic acid (MTPA) esters (Mosher esters) of **1** and **2** were prepared and numbered **1s**, **1r** (from **1**) and **2s**, **2r**, respectively. COSY ^{1}H-nmr analyses of these derivatives were then performed. The ^{1}H-nmr chemical shift data of **1s**, **1r**, **2s**, and **2r** showed that the absolute configuration at C-4 of **1** and **2** is *R* (Table 3). This result is identical to all acetogenins examined so far that possess an OH at C-4.

Similarly, the Mosher ester data (Table 4) allowed the absolute stereochemical assignment of the carbinol centers adjacent to the mono-THF ring in **1** and **2** as C-15*R* and C-20*S*. The assignment of the absolute stereochemistry of the asymmetric centers at C-10/C-11 of the vicinal diol of **1** and **2** could not be achieved by direct application of the Mosher ester method.

Annomuricins A and B [**1** and **2**] were significantly bioactive in the BST assay and were also cytotoxic (seven-day MTT assays) to human solid tumor cell lines in culture (Table 5). In comparison with annonacin (5), annomuricins A [**1**] and B [**2**] have one

TABLE 3. ^{1}H-Nmr Chemical Shifts for the Determination of the Absolute Configuration at C-4 of the Penta (*S*)- and (*R*)-MTPA Esters of **1** and **2**.

MTPA ester of	H_2C-5	HC-4	H_2C-3		HC-33	HC-34	H_3C-35	Configuration
1s δ (*S*)	1.59	5.29	2.57	2.52	6.73	4.86	1.27	4*R*
1r δ (*R*)	1.58	5.34	2.65	2.56	6.95	4.90	1.29	
Δ δ	+0.01	−0.05	−0.08	−0.04	−0.22	−0.04	−0.02	
2s δ (*S*)	1.57	5.29	2.57	2.52	6.72	4.85	1.27	4*R*
2r δ (*R*)	1.56	5.35	2.65	2.56	6.95	4.89	1.29	
Δ δ	+0.01	−0.06	−0.08	−0.04	−0.23	−0.04	−0.02	

TABLE 4. ^{1}H-Nmr Chemical Shifts for the Determination of the Absolute Configurations at C-15 and C-20 of the Penta (*S*)- and (*R*)-MTPA Esters of **1** and **2**.

MTPA Ester of	H_2C-14	HC-15	HC-16	H_2C-17/18	HC-19	HC-20	H_2C-21	Configuration
1s δ (*S*)	1.53	5.21	3.93	1.85	3.89	4.94	1.51	15*R*
	1.46			1.65			1.43	20*S*
1r δ (*R*)	1.48	5.26	3.97	1.82	3.70	4.86	1.54	
	1.42			1.56			1.47	
Δ δ	pos.	neg.	neg.	pos.	pos.	pos.	neg.	
2s δ (*S*)	1.53	5.22	3.93	1.86	3.90	5.02	1.51	15*R*
	1.46			1.65			1.43	20*S*
2r δ (*R*)	1.48	5.27	3.98	1.83	3.70	4.90	1.54	
	1.42			1.57			1.47	
Δ δ	pos.	neg.	neg.	pos.	pos.	pos.	neg.	

additional OH group, but their cytotoxic effects on these tumor cell lines are notably reduced (18). However, if a second THF ring were to be formed, using the vicinal diol groups in **1** and **2**, the cytotoxicities of the resultant bis-THF products would likely increase because the bis-THF compounds are more bioactive than the mono-THF acetogenins (18,19). This trend was substantiated by the acetonides **1c** and **2c**, which were one- to threefold more cytotoxic and three times more toxic in the BST than **1** and **2** (Table 5). The presence of the second ring might account for the increased activities. The greater susceptibility of the colon cell line (HT-29) to **2** and **2c**, vs. **1** and **1c** (Table 5), suggests that the stereochemistry of the diols can play a role in controlling selectivity toward specific cell lines and tumor types.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra (films) were measured on a Perkin-Elmer 1420 ir spectrometer. ^{1}H-Nmr, COSY, and ^{13}C-nmr spectra were obtained on a Varian VXR-500S spectrometer. Ms spectra were obtained on a Kratos MS-50 spectrometer. Hplc was carried out using a Dynamax software system and a Si gel (8 μm) column (250×21 mm) equipped with a Rainin UV-1 detector. Analytical tlc was performed on Si gel plates (0.25 mm) developed with $CHCl_3$-MeOH (10:1) and hexane-Me_2CO (4:3) and ether-Me_2CO (3:2), and visualized with 5% phosphomolybdic acid in EtOH.

PLANT MATERIAL.—Fresh leaves of *Annona muricata* L. were collected from fruit-producing trees growing in the experimental orchard of Bandung Institute of Technology. The leaves were air dried and pulverized through an 8-mm sieve in an electric mill.

TABLE 5. Bioactivity of **1** and **2** and Their Acetonide Derivatives (**1c** and **2c**).[a]

Compound	BST[b] 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)
1[f]	6.25×10^{-1}	3.30×10^{-1}	>1.0	>1.0
2[f]	6.87×10^{-1}	1.59×10^{-1}	>1.0	4.35×10^{-1}
1c[g]	1.31×10^{-1}	1.58×10^{-4}	2.78×10^{-4}	>1.0
2c[g]	1.80×10^{-1}	3.41×10^{-4}	1.41×10^{-4}	9.33×10^{-2}
Adriamycin[g,h]	—	1.01×10^{-2}	1.26×10^{-1}	2.75×10^{-2}

[a]Values in different runs were within one order of magnitude of each other.
[b]Brine shrimp lethality test.
[c]Human lung carcinoma.
[d]Human breast carcinoma.
[e]Human colon adenocarcinoma.
[f,g]Same cytotoxicity runs.
[h]Positive control standard.

BIOASSAYS.—The extracts, fractions, and isolated compounds were routinely evaluated for lethality to brine shrimp larvae (BST) (10,11). Seven-day cytotoxicity against human solid tumor cell lines was measured at the Cell Culture Laboratory, Purdue Cancer Center, for the A-549 lung carcinoma (22), MCF-7 breast carcinoma (23), and HT-29 colon adenocarcinoma (24).

EXTRACTION AND ISOLATION.—The pulverized leaves (2.0 kg) were repeatedly percolated with 95% EtOH to yield 386 g of an extract (F001), on removal of solvent. F001 was partitioned between $CHCl_3$-H_2O (1:1) to yield the H_2O-soluble fraction (F002, 260 g) and the $CHCl_3$-soluble fraction (F003, 100 g). F003 was then partitioned between 90% aqueous MeOH-hexane (1:1) to yield a hexane-soluble fraction (F006, 39 g) and an aqueous MeOH-soluble fraction (F005, 60 g). All fractions were subjected to the BST, with the most active fraction being F005 (BST, LC_{50}=0.17 μg/ml). F005 (16 g) was applied to a column of Si gel (230–400 mesh), packed in a hexane slurry, and developed, with hexane containing gradually increasing amounts of EtOAc and finally with MeOH, to yield fraction Nos. 1–154. The BST active fraction No. 128 (BST, LC_{50}= 1.25 μg/ml) was further subjected to repeated flash chromatography to yield crude compounds **1** and **2**; each was then purified by hplc, eluted with hexane-MeOH (90:1, flow rate 10 ml/min), to afford the two colorless, amorphous powders **1** and **2**. Using the same methods, six known compounds (gigantetrocin A, annonacin-10-one, muricatetrocins A and B, annonacin, and goniothalamicin) were isolated from bioactive fraction Nos. 86–127.

Annomuricin A [**1**].—White powder (8 mg); $[\alpha]^{22}D$ −6.4° (c=0.0025), ir ν max (film) 3431 (br OH), 2919, 2849, 1734, 1699, 1073 cm^{-1}; uv λ max (MeOH) 220 nm (ε 3.1×10^3); hrfabms (glycerol) *m/z* $[MH]^+$ 613.4691 for $C_{35}H_{65}O_8$ (calcd 613.4679); cims *m/z* 613 (6), 595 (3), 577 (3), 559 (30), 541 (12), 523 (2), 395 (1), 353 (6), 325 (6), 271 (3), 269 (5), 253 (5), 241 (39), 223 (6), 205 (4), 199 (4); eims *m/z* 341 (2), 325 (11), 271 (3), 269 (2), 241 (17), 223 (2), 205 (2), 199 (4), and 141 (7); ^{1}H-nmr data ($CDCl_3$, 500 MHz), see Table 1; ^{13}C-nmr data ($CDCl_3$, 125 MHz), see Table 1.

Annomuricin B [**2**].—White powder (7 mg); $[\alpha]^{22}D$ −11.7° (c=0.0064); ir ν max (film) 3430 (br, OH), 2921, 2851, 1733, 1684, 1075 cm^{-1}; uv λ max (MeOH) 220 nm (ε 3.0×10^3); hrfabms (glycerol) *m/z* $[MH]^+$ 613.4694 for $C_{35}H_{65}O_8$ (calcd 613.4679); cims *m/z* $[MH]^+$ 613 (100), 595 (11), 577 (19), 559 (3), 541 (0.1) and 523 (2); ^{1}H-nmr data ($CDCl_3$, 500 MHz), see Table 1; ^{13}C-nmr data ($CDCl_3$, 125 MHz), see Table 1.

TMSi DERIVATIZATIONS.—Samples (1 mg of **1** or **2**) were treated with 20 μl of *N,O-bis*-(trimethylsilyl)-acetamide and 2 μl of pyridine and heated at 70° for 30 min to yield the respective penta-TMSi derivatives (**1b** and **2b**); eims of **1b** *m/z* 701 (14), 631 (45), 611 (38), 587 (6), 541 (32), 521 (26), 497 (26), 487 (5), 485 (10), 397 (8), 385 (100), 341 (30), 295 (18), 271 (54), 213 (35); eims of **2b** *m/z* 701 (1), 631 (14), 611 (8), 587 (4), 541 (14), 521 (9), 497 (17), 487 (3), 485 (4), 397 (55), 385 (89), 341 (39), 295 (17), 271 (96), 213 (36); hrfabms (glycerol) *m/z* 385.2239 [$C_{19}H_{37}O_4Si_2$] (**1b** and **2b**) (calcd 385.2230).

PREPARATION OF ACETONIDE DERIVATIVES.—Compound **1** or **2** (1.5 mg) was added into 0.5 ml of HCl/Me_2CO (0.7 μg HCl in 1 ml Me_2CO) and left overnight at room temperature; the mixture was dried *in vacuo*, and the ^{1}H-nmr chemical shifts of the acetonides (**1c** and **2c**) were recorded (see Table 1).

PREPARATION OF *S* AND *R* MTPA-ESTER DERIVATIVES.—To **1** or **2** (1.0 mg in 0.5 ml of CH_2Cl_2) were sequentially added 0.2 ml pyridine, 0.5 mg 4-(dimethylamino) pyridine, and 25 mg of (*R*)-(−)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride. The mixture was left at room temperature for 5–6 h and purified over a micro-column (0.6×6 cm) of Si gel (200–400 mesh) eluted with 3–4 ml of CH_2Cl_2; the eluate was washed using 1% $NaHCO_3$ (5–6 ml) and H_2O (2×6 ml); the eluate was dried *in vacuo* to give the *S*-Mosher esters (**1s** and **2s**). Using (*S*)-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride gave the *R*-Mosher esters (**1r** and **2r**). Their pertinent ^{1}H-nmr chemical shifts are given in Tables 3–5.

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