Tamosterone Sulfates: A C-14 Epimeric Pair of Polyhydroxylated Sterols from a New Oceanapiid Sponge Genus

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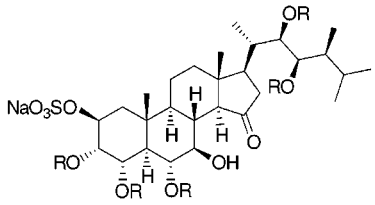
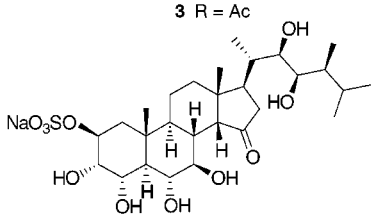
Two novel, C-14 epimeric polyhydroxylated 15-keto steroid sulfates, tamosterone sulfate and 14β-tamosterone sulfate (**1** and **2**), were isolated from a sponge belonging to a new genus collected in Yap, Federated States of Micronesia. Their structures were assigned by analysis of spectroscopic data and chemical conversions. 14β-Tamosterone sulfate (**2**) has the rare naturally occurring C/D cis ring fusion and is the less stable epimer based on equilibration studies. Both compounds possess side-chain substitution patterns not observed previously in marine sterols.

A diverse array of novel steroids has been isolated from marine invertebrates, and sponges have been the source of many of these.[1] Varied and extensive hydroxylation patterns as well as unique side chains have been the distinguishing characteristics of many of these novel steroids. A particularly uncommon feature in the extensive list of novel marine sterols is the cis C/D ring fusion. All five marine sterols possessing this feature have been isolated from sponges.[2−5] These include contignasterol isolated from the sponge *Petrosia contignata*,[2] xestobergsterols A−C from *Xestospongia bergquistia*[3] and an *Ircinia* sp.,[4] and haliclostanone sulfate from a *Haliclona* sp.[5] In the course of our ongoing investigation of marine organisms,[6] we investigated a sponge of a new genus collected in Yap, Federated States of Micronesia. The sponge represents a new genus of the haplosclerid family Oceanapiidae, *Haliclona pellasarca* de Laubenfels 1934 being the suggested genus holotype (Van Soest, 1980). From this sponge, we isolated two new sterol sulfates, tamosterone sulfate and 14β-tamosterone sulfate[7] (**1** and **2**, respectively). Sterol **2** has the uncommon 14β configuration. In this paper, we report the isolation and structure elucidation of these new compounds.

The MeOH and MeOH−CH_2Cl_2 (1:1) extracts of freshly thawed specimens were concentrated and subjected to solvent partitioning[8] to give hexane-, CH_2Cl_2-, and *n*-BuOH-soluble fractions. Metabolites **1** and **2** were obtained from the *n*-BuOH-soluble fraction by a combination of Si gel chromatography and C_{18} reversed-phase HPLC as described in the Experimental Section.



1 R = H
3 R = Ac



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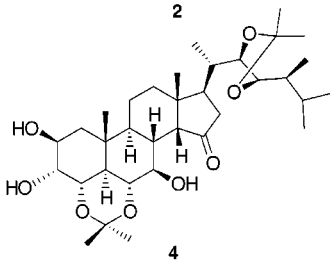

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Tamosterone sulfate (**1**) was obtained as an amorphous solid, and its molecular formula was established by HRFABMS (see the Experimental Section) and by NMR data (Table 1) to be $C_{28}H_{47}NaO_{11}S$. The IR spectrum of **1** revealed bands at 3370 (br), 1716, 1260 cm^{-1} indicative of hydroxyl, ketone, and sulfate groups. A ^{13}C NMR signal at δ 221.7 (s) also denoted the presence of a ketone. The sulfate and ketone functionalities and the steroid nucleus accounted for all the degrees of unsaturation. Hence, there were no cyclic ethers present, and all oxymethine carbons were substituted with hydroxyl groups except for one that bore a sulfate group. The ^1H NMR spectrum of **1** contained two methyl singlets at δ 0.84 and 1.06, assigned to Me-18 and Me-19, respectively, and four methyl doublets at δ 0.84, 0.93, 0.97, 0.98, attributed to

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Table 1. NMR Data for 1 and 2 in CD_3OD

	1		2	
position	^{13}C (mult)[a]	^1H (mult, *J* in Hz)[b]	^{13}C (mult)	^1H (mult, *J* in Hz)
1α	38.1 (t)	1.49 (m)	38.1 (t)	1.43 (dd, 14.8, 3.4)
1*β*		2.03 (dd, 14.1, 1.3)		2.04 (m)
2	78.4 (d)	4.58 (q, 2.7)	78.4 (d)	4.57 (q, 3.0)
3	71.8 (d)	3.98 (t, 2.7)	71.7 (d)	3.99 (t, 2.7)
4	71.3 (d)	4.21 (dd, 10.8, 2.7)	71.3 (d)	4.22 (dd, 10.8, 2.7)
5	45.1 (d)	1.69 (t, 10.7)	45.1 (d)	1.67 (t, 10.8)
6	76.2 (d)	3.85 (dd, 11.5, 8.1)	76.8 (d)	3.72 (dd, 10.8, 8.8)
7	79.3 (d)	3.33 (t, 8.8)	75.4 (d)	4.43 (dd, 10.8, 8.8)
8	40.5 (d)	1.80 (q, 10.1)	40.1 (d)	1.68 (m)
9	53.1 (d)	0.97 (m)	48.0 (d)	1.04 (m)
10	38.7 (s)		39.0 (s)	
11α	21.9 (t)	1.68 (m)	22.4 (t)	1.53 (m)
11*β*		1.34 (m)		1.29 (m)
12α	40.8 (t)	1.49 (m)	37.8 (t)	1.28 (m)
12*β*		2.15 (m)		1.39 (m)
13	44.6 (s)		42.9 (s)	
14	68.5 (d)	2.29 (d, 9.4)	52.7 (d)	2.73 (br s)
15	221.7 (s)		223.2 (s)	
16α	42.1 (t)	2.73 (dd, 18.9, 8.1)	40.2 (t)	2.35 (dd, 19.5, 10.1)
16*β*		1.96 (dd, 18.9, 10.8)		2.83 (br d, 19.5)
17	49.5 (d)	2.13 (m)	49.5 (d)	1.79 (m)
18	13.6 (q)	0.84 (s)	19.6 (q)	1.20 (s)
19	16.7 (q)	1.06 (s)	16.9 (q)	1.02 (s)
20	38.1 (d)	1.64 (m)	35.0 (d)	2.04 (m)
21	13.1 (q)	0.98 (d, 6.7)	14.0 (q)	0.956 (d, 6.7)
22	75.3 (d)	3.40 (d, 8.1)	74.7 (d)	3.79 (dd, 7.4, 1.4)
23	74.2 (d)	3.70 (dd, 8.1, 2.0)	75.3 (d)	3.61 (dd, 7.4, 2.7)
24	41.8 (d)	1.17 (ddq, 2.0, 6.7, 6.7)	41.9 (d)	1.30 (m)
25	31.7 (d)	1.64 (m)	31.8 (d)	1.64 (m)
26	21.1 (q)	0.93 (d, 6.7)	20.7 (q)	0.92 (d, 6.7)
27	21.4 (q)	0.97 (d, 6.7)	21.6 (q)	0.963 (d, 6.7)
28	10.8 (q)	0.84 (d, 6.7)	11.2 (q)	0.88 (d, 6.7)

[a] ^{13}C NMR at 125 MHz, referenced to CD_3OD (*δ* 49), multiplicities inferred from DEPT and HMQC experiments. [b] ^1H NMR at 500 MHz, referenced to residual solvent CD_3OD (*δ* 3.30).

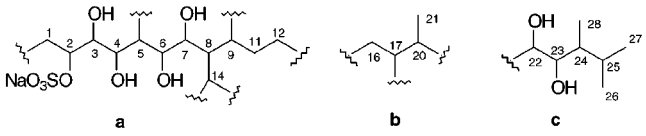

Figure 1. Partial structures **a**−**c**.

the methyl groups on the side chain. Also present were seven oxymethine signals at *δ* 4.58 (H-2), 3.98 (H-3), 4.21 (H-4), 3.85 (H-6), 3.33 (H-7), 3.40 (H-22), and 3.70 (H-23), which were associated with ^{13}C signals at *δ* 78.4 (C-2), 71.8 (C-3), 71.3 (C-4), 76.2 (C-6), 79.3 (C-7), 75.3 (C-22), and 74.2 (C-23), respectively, on the basis of HMQC data. Interpretation of the COSY and relayed coherence transfer-COSY (RCT-COSY) spectra of **1** resulted in formulation of partial structures **a**−**c** (Figure 1). All the protonated carbon-13 signals were assigned from HMQC data, and combining this with HMBC data enabled us to establish the planar structure of **1**. HMBC correlations from Me-19 (*δ* 1.06) to *δ* 38.1 (C-1), 45.1 (C-5), 53.1 (C-9), and 38.7 (C-10) established the connection of C-1, C-5, C-9, and C-19 to C-10, and this formed rings A and B of the steroid nucleus. The connectivity of C-12, C-14, C-17, and C-18 to C-13 was based on HMBC cross-peaks between Me-18 protons (*δ* 0.84) and *δ* 40.8 (C-12), 44.6 (C-13), 68.5 (C-14), and49.5 (C-17). HMBC correlations between the ketone carbon (*δ* 221.7) and H-14 (*δ* 2.29) and H-16 (*δ* 2.73 and 1.96) confirmed the location of the ketone at C-15. The connection between partial structures **b** and **c** was based on long-range coupling between Me-21 (*δ* 0.98) and C-22 (*δ* 75.3).

The locus of the sulfate group was assumed to be at C-2 on the basis of the downfield chemical shift of both C-2 (*δ* 78.4) and H-2 (*δ* 4.58) compared to the remaining oxymethines. This assumption was verified by the ^1H NMR data of pentaacetate **3** prepared by reaction of **1** with acetic anhydride in pyridine. The ^1H NMR spectrum of **3** contained only five acetate methyl singlets (*δ* 2.09, 2.06, 2.04, 2.00, 1.89), indicating that only five out of six secondary alcohols in compound **1** were acetylated. This was consistent with a negative FABMS ion peak at *m*/*z* 801 $[M - Na]^-$. In the ^1H NMR spectrum of **3**, oxymethine protons H-3 (*δ* 5.58), H-4 (*δ* 5.31), H-6 (*δ* 5.13), H-22 (*δ* 5.02), and H-23 (*δ* 5.34) resonated farther downfield than the corresponding protons in compound **1**, while H-2 (*δ* 4.65) and H-7 (3.37) resonated at the same positions ($\Delta\delta < 0.07$ ppm) as in **1**. Hence, the sulfate was located at C-2, and further evidence for this was provided by the loss of C-2 sulfate group during the preparation of acetonide **4** from **2**. The hydroxyl group at C-7 was not acetylated, presumably due either to steric hindrance from the ketone or hydrogen bonding to it.

The relative stereochemistry of **1** was established from coupling constants and NOESY data. Diagnostic coupling constants (Table 1) indicated that H-2 and H-3 were equatorially oriented and that H-4, H-5, H-6, H-7, H-8, and H-14 were all axially oriented. The H-9 signal was observed as a multiplet at *δ* 0.97, but a 10.1 Hz coupling between H-8 and H-9 could be assigned on the basis of the coupling observed for H-8 (*δ* 1.80, q, $J = 10.1$ Hz); hence, H-9 is axial. Observation of the following NOESY correlations unequivocally defined the usual chair conformation and trans ring fusions for **1**: Me-19/H-4, H-6, H-8, and H-11*β*; Me-18/H-11*β*, H-12*β*, H-8, and H-16*β*; H-7/H-5, H-9, and H-14. NOESY cross-peaks between H-17 (*δ* 2.13) and H-12α (*δ* 1.49) and H-14 (*δ* 2.29) were indicative of an α-oriented H-17, which was consistent with the coupling constants ($J = 10.8$, 8.1 Hz) between H-17 and the protons at C-16.

The stereochemistry of the stereogenic centers in the side chain was strongly suggested to be 21*S*,22*R*,23*R*,24*S* on the basis of the ^1H NMR chemical shifts (Table 1) of the side chain's methyl groups and the coupling constants between H-22 and H-23 ($J = 8.1$ Hz) and between H-22 and H-20 ($J \approx 0$ Hz). These data were virtually identical to those of many natural[9] and synthetic[10] brassinosteroids bearing this same side chain. This suggestion was supported by observation of the following significant NOESY correlations: H-17 (*δ* 2.13)/Me-21 (*δ* 0.98), H-22 (*δ* 3.40)/H-16*β* (*δ* 1.96), H-22/H-20 (*δ* 1.64), H-22/Me-28 (*δ* 0.84), H-23 (*δ* 3.70)/Me-21, and H-23/H-24 (*δ* 1.17).

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The second new metabolite, 14β-tamosterone sulfate (**2**), was also obtained as an amorphous powder. The molecular formula $C_{28}H_{47}NaO_{11}S$ established by HR-FABMS and NMR data (Table 1) was identical to that of **1**, indicating that the compounds were isomers. The ^{1}H and ^{13}C NMR data (Table 1) of **2** closely resembled that of **1** except for some signals surrounding C-14. COSY, RCT-COSY, HMQC, and HMBC data provided evidence for the same planar structure for **2** as that of **1**. The difference between **1** and **2** was traced to the difference in the stereochemistry of H-14: 14β in **2** vs 14α in **1**. This was evident from the small coupling constant for H-14 (δ 2.73, br s), and NOESY correlations between H-14 and Me-18 and between H-14 and H-8. The downfield shift (Δ = 6 ppm) of C-18 in the ^{13}C NMR spectrum of **2** relative to that observed for **1** was also in agreement with the cis C/D ring junction.[2−5] Therefore, the structure of the *steroid nucleus* in **2** is identical with that of haliclostanone sulfate, which was recently reported from an Indo-Pacific *Haliclona* sponge.[5]

Reaction of **2** with acetone in the presence of a trace of iodine led to the formation of **4** (74%), which possessed two ketals but no sulfate group as judged by ^{1}H NMR, ^{13}C NMR, and HRFABMS data (see the Experimental Section). The upfield shifts of H-2, H-3, and Hs-1 confirmed that the site of the sulfate group in **2** was at C-2. That one of the two ketals was formed on the side chain was indicated by the downfield shifts of H-22 (δ 4.02 vs 3.79 in **2**) and H-23 (δ 3.82 vs 3.61 in **2**). NOESY correlations between one of the acetonide methyls at δ 1.49 and both H-4 (δ 4.24) and H-6 (δ 3.68) revealed that the second ketal was formed with hydroxyls at C-4 and C-6, although the chemical shifts of H-4 and H-6 in **2** and **4** were essentially identical.

The stereochemistry at each of the stereogenic centers in the side chain of **2** was determined to be the same as in **1**. This was confirmed by interconverting **1** and **2** by treating each pure compound with $NaOCH_3$ in CH_3OH. An approximately 2:1 mixture of **1**:**2** was obtained in these equilibration experiments. This was somewhat surprising in view of the reported thermodynamic preference for the C/D cis fusion for several semisynthetic[11a] 15-keto steroids and a 15-ketobufadienolide.[11b] It is, however, consistent with the report by Barton and Laws[12] that 3β-acetoxyergost-22-en-15-one (C/D trans) did not epimerize under strongly basic conditions. Allinger et al.[11a] argued that the preference for C/D cis fusion in the case of a 15-keto-17-carbomethoxy steroid might be due to the fact that the planar carbomethoxy group does not cause as much steric repulsion with Me-18 in the C/D cis arrangement as does the conventional saturated steroid side chain. In the case of **1** and **2**, attractive/repulsive interactions between the 7β-OH and the 15-keto group may also play a role in determining the epimer ratio.

Sterol **2** is only the sixth example of the rare class of naturally occurring sterols containing a 14β hydrogen.[5] It is also noteworthy that **1** and **2** bear a side-chain substitution that is unprecedented in marine steroids reported to date. To our knowledge, brassinosteroids,[13] a class of plant growth-promoter isolated from plants, represent the only other steroids bearing this substituted side chain.

Co-occurrence of compounds **1** and **2** is interesting because the 14β steroids contignasterol, xestobergsterols A−C, and haliclostanone sulfate were reported without mention of the occurrence of their 14α epimers in the same extracts. The isolated ratio (aproximately 1:5) of compounds **1** and **2** is quite different from the ratio obtained by equilibration studies. This would seem to rule out the possibility that **2** is an artifact derived by epimerization of **1** during the isolation procedures.

Experimental Section

General Experimental Procedures. All solvents were redistilled. Merck Si gel 60 (230−240 mesh) was used for vacuum flash chromatography. HPLC was conducted using an RI detector and a Spherex 5 C-18 column. All proton NMR spectra were measured at 500 MHz and carbon-13 NMR spectra at 125 MHz.

Animal Material. The specimen was collected from Barge Reef, east of Tamil Channel on the outer reef of Yap, Federated States of Micronesia, on August 8, 1995, from a depth of 33 m. The sponge is hemispherical, 15 cm diameter, 8 cm high, brownish red externally, and cream-tan internally. The texture is very soft, and the sponge crumbles easily. The surface is covered with a parchment-like skin. Oscules are grouped on the sponge apex. The choanosome of the sponge is a complex isodictyal reticulation of oxeas (150−170 μm), with a regular surface of spicule tufts echinated by smaller oxeas (60−100 μm). Toxa, 30 μm long, are scattered in the choanosome. The sponge and its skeletal architecture bear a strong resemblance to *Haliclona pellasarca* de Laubenfels (1934) suggested as the genus holotype for a new genus of Petrosiidae by Van Soest (1980). However, the sponge is closer morphologically to oceanapiid genera *Biminia*, in that it possess several size categories of toxas, and *Pellina*, in that it has visible parchment-like growth rings, a delicate reticulation of uniformly small oxeas, and occasional "stringy" tracts between these rings. Within the deeper choanosome spicules form palisades around cavities. The sponge represents a new genus of the family Oceanapiidae (order Haplosclerida). A voucher specimen has been deposited in the Natural History Museum, London (BMNH 1998.5.15.1) and University of Oklahoma (40YA95).

Extraction and Isolation. Freshly thawed specimens of the sponge (987 g wet wt; 98 g dry wt after extraction) were minced and soaked in MeOH (2 × 1.5 L) followed by MeOH−CH_2Cl_2 (1:1) (2 × 1.5 L). All extracts were combined after removal of solvents in vacuo and subjected to solvent partitioning as described previously.[8] This gave, after evaporation of solvents in vacuo, hexane (3.0 g), CH_2Cl_2 (2.2 g), and *n*-BuOH (11.8 g) solubles. A portion (6 g) of the *n*-BuOH fraction that showed interesting ^{1}H NMR signals was subjected to chromatography on silica gel using a stepwise gradient of MeOH and CH_2Cl_2 as eluent. One-fourth of a fraction eluted with 50% MeOH−CH_2Cl_2 was further separated by reversed-phase HPLC on a C_{18} column using 50% H_2O−MeOH as eluent to give compound **1** (11.4 mg; 0.078% of dry specimen weight) and compound **2** (53.2 mg; 0.36%).

Tamosterone sulfate (1): amorphous solid; $[\alpha]_D$ +34.4° (*c* 0.30, MeOH); IR (NaCl) ν_{max} 3370 (br), 1716, 1260, 1210 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; assignments were made by interpretation of COSY, RCT-COSY, HMQC, HMBC,

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and NOESY data; HRFABMS *m/z* 637.2653 $[M + Na]^+$ (Δ −1.9 mmu), 615.2850 $[M + H]^+$ (Δ −3.5 mmu).

14*β*-Tamosterone sulfate (2): amorphous solid; $[\alpha]_D$ +5.7° (*c* 0.81, MeOH); IR (NaCl) ν_{max} 3445 (br), 1723, 1260, 1206 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; assignments were based on COSY, RCT-COSY, HMQC, HMBC, and NOESY data; HRFABMS *m/z* 615.2851 $[M + H]^+$ (Δ −3.6 mmu).

Tamosterone Sulfate Pentaacetate (3). A 2 mg portion of **1** was dissolved in a mixture 200 μL of pyridine and 200 μL of Ac_2O. The mixture was kept overnight at ambient temperature and then diluted with ice−water to destroy excess Ac_2O. The resulting solution was extracted with CH_2Cl_2. The CH_2Cl_2 portion was washed with water, and after the solvent was evaporated pentaacetate **3** was recovered (90% pure): ^1H NMR ($CDCl_3$, 500 MHz) δ 6.30 (br s, OH), 5.58 (br s, H-3), 5.34 (d, J = 9.0 Hz, H-23), 5.31 (d, J = 10.5 Hz, H-4), 5.13 (dd, J = 11.5, 8.5 Hz, H-6), 5.02 (d, J = 9.0 Hz, H-22), 4.65 (br s, H-2), 3.37 (t, J = 9.0 Hz, H-7), 2.09, 2.06, 2.04, 2.00, 1.89 (each 3H, s, five acetate methyls), 1.15 (s, Me-19), 1.09 (d, J = 6.5 Hz), 0.94 (d, J = 6.5 Hz), 0.92 (d, J = 7.0 Hz), 0.90 (d, J = 7.0 Hz) (each 3H, Me-21, 26, 27, and 28), 0.82 (s, Me-18); negative ion FABMS *m/z* 801 $[M - Na]^-$.

Compound 4. A catalytic amount of iodine was added to a stirred solution of **2** (5 mg, 0.008 m moles) in acetone (5 mL).[14] The mixture was refluxed for 4 h. After being cooled to room temperature, the mixture was treated with 0.5 M aqueous sodium thiosulfate ($Na_2S_2O_3$) until colorless and concentrated in vacuo to remove the acetone. The resultant solution was diluted with water and then extracted with CH_2Cl_2 (2 × 5 mL). The organic layer was separated, washed with water (2 × 5 mL) and 10% aqueous NaCl solution (5 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford 3.6 mg of **4** (74.4% yield): IR (NaCl) ν_{max} 3431 (br), 1734, 1466, 1382, 1260 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 1.68 (br d, J = 14.1 Hz, H-1*β*), 1.39 (m, H-1α), 3.94 (q, J = 3.0 Hz, H-2), 3.75 (t, J = 2.7 Hz, H-3), 4.24 (dd, J = 11.5, 3.0 Hz, H-4), 1.57 (t, J = 11.5 Hz, H-5), 3.68 (dd, J = 11.5, 8.1 Hz, H-6), 4.47 (dd, J = 10.8, 8.8 Hz, H-7), 1.65 (m, H-8), 1.07 (m, H-9), 1.49 (m, H-11α), 1.30 (m, H-11*β*), 1.39 (m, H-12*β*), 1.26 (m, H-12α), 2.82 (br s, H-14), 2.71 (br d, J = 19.5 Hz, H-16*β*), 2.32 (dd, J = 19.5, 10.1 Hz, H-16α), 1.81 (br d, J = 10.1 Hz, H-17), 1.18 (s, Me-18), 1.07 (s, Me-19), 2.12 (m, H-20), 0.99 (d, J = 6.5 Hz, Me-21), 4.02 (br d, J = 8.7 Hz, H-22), 3.82 (dd, J = 8.7, 2.7 Hz, H-23), 1.40 (m, H-24), 1.68 (m, H-25), 0.92, 0.94 (each d, J = 6.5 Hz, Me-26, Me-27), 0.91 (d, J = 7.0 Hz, Me-28), 1.49, 1.40, 1.29, 1.25 (each s, acetonide methyls), assignments were based on COSY and NOESY data; ^{13}C NMR (CD_3OD, 125 MHz) δ 222.6, 108.8, 99.8, 80.7, 79.9, 73.4, 72.6, 72.0 (2C), 67.3, 52.8, 42.7, 41.6, 40.4, 40.3, 39.6, 39.4, 38.2, 36.8, 32.9, 32.8, 30.7, 30.6, 27.4, 27.2, 22.3, 20.9, 20.5, 19.7, 19.0, 16.5, 13.8, 10.5; HRFABMS *m/z* $[M + H]^+$ 593.4066 (Δ −1.3 mmu).

Epimerization of 2 and 1. A 12 mg portion of **2** was added to a solution of $NaOCH_3$ (~10 mg) in MeOH (3 mL). The mixture was kept overnight at room temperature, and then 3 mL of ice−water was added. After most of the MeOH was evaporated under a stream of N_2, the resulting solution was extracted with *n*-BuOH. The *n*-BuOH-soluble material, after removal of solvent, was subjected to C_{18} reversed-phase HPLC using 50% H_2O−MeOH as eluent to give **1** (6.7 mg) and **2** (3.5 mg). Tamosterone sulfate **(1)** (1.8 mg) was processed in the same manner. After workup, the *n*-BuOH-soluble material was analyzed by reversed-phase HPLC using the same conditions as used for the above analysis. The same retention times were observed, and the same ratio (approximately 2:1) of compounds **1** and **2** was noted as was obtained from the reaction of **2** with $NaOCH_3$ in MeOH.

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Supporting Information Available: ^1H and ^{13}C NMR spectra for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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