Ecteinascidins: Putative Biosynthetic Precursors and Absolute Stereochemistry

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Abstract: New bioactive ecteinascidins (Et's) 597 (**1**), 583 (**2**), 594 (**3**), and 596 (**4**)—putative biosynthetic precursors of previously described Et's [e.g., Et 743 (**5**)]—were isolated from the Caribbean tunicate *Ecteinascidia turbinata.* Structures assigned to these compounds based on spectroscopic data represent a novel series of Et's with L-cysteine or its α-oxo analog as unit C. The absolute configuration of the L-Cys unit of **1** was assigned by chiral GC, while a 2D ROESY (rotating-frame Overhauser enhancement spectroscopy) spectrum of its acetyl derivative **1a** completed the assignment of the stereochemistry of **1** as 1*R*,2*R*,3*R*,4*R*,11*R*,13*S*,21*S*,1′*R*.

In the present paper we report new ecteinascidins (Et's) **1**−**4** (Et's 597, 583, 594, and 596, respectively; Chart 1). Et's are exceedingly potent antitumor agents first isolated by Holt[1a−c] from the marine tunicate *Ecteinascidia turbinata*, following earlier decade-long efforts.[1d−f] Previously reported Et's 743 (**5**), 729 (**6**), 736 (**7**), and 722 (**8**) show promising efficacy in vivo, including activity against P388 murine leukemia, B16 melanoma, M5076 ovarian sarcoma, Lewis lung carcinoma, and several human tumor xenograft models in nude mice.[1g] With advanced stage MX-1 mammary tumor xenografts, 9 of 10 mice were tumor free on day 23 following treatment with **5**, and with early stage MX-1 xenografts, 10 of 10 were tumor free (Table 1). The most abundant analogue, **5**, is now in phase I clinical trials as an anticancer agent in three European countries and in the United States.[2]

The structures of Et's 743 (**5**), 729 (**6**), 745 (**9**), 759 B (**10**), and 770 (**11**) were assigned primarily by spectroscopic, especially FABMS and NMR, studies. Pseudomolecular formulas $(M + H - H_2O)^+$ of Et's were assigned by HRFABMS and ESCA (electron spectroscopy for chemical analysis), which identified the sulfur,[1a] and molecular formulas $(M - H)^-$ assigned by negative ion HRFABMS, which required an extra water molecule.[3a] The three tetrahydroisoquinoline units and their aromatic ring (A, B, C) substituents were identified by Holt[1a] and these were combined into partial formulas,[1c] the substituent patterns of which at C-5 to C-8 and C-6′ to C-8′ were later corrected, first by Wright et al.[3b] and also by us,[3a] and incorporated into consensus structures based on the correct formulas.[3a,b] The relative stereochemistry of Et's for most of the stereocenters in the A−B unit was assigned by NMR data,[3a,b,d] and crystal structures of some Et analogues completed the unambiguous assignment of relative stereochemistry, especially at the difficult quaternary C-1′.[1g,3c] Although the X-ray diffraction data did not assign the absolute stereochemistry, it was assumed to be the same as in safracin A, *R* at C-1′.[3a,b] The present study confirms the absolute stereochemistry suggested.

Table 1. Antitumor Activity of Et 743 (**5**) against Early Stage MX-1 Breast Tumor Xenografts[a]

dose (μg/kg)	schedule	weight loss, %	no. tumor free (23 days)[b]	*T*/*C* (%)[c]
60	iv QD × 5	5.6 (21 days)	10	0 (15 days)
40		1.4 (12 days)	7	0 (12 days)
27		no loss	1	4 (21)

[a] Taken from G. Faircloth, et al., Proceedings of the International Symposium on Changes in the Treatment of Breast Cancer, Madrid, June 5−6, 1995. [b] Ten mice were used. [c] *T*/*C* = 100% × treated tumor wt/control tumor wt; *T*/*C* < 40 was considered active.

The previously reported ecteinascidins are composed either of three tetrahydroisoquinoline subunits A−C or two tetrahydroisoquinoline units (A,B) plus one tetrahydro-β-carboline unit (C) (Chart 1). The carbon and nitrogen framework of units A−B of the Et's is the same as that of the saframycins and safracins, antitumor agents first isolated from cultured *Streptomyces* species.[4] Compounds related to the saframycins which have the same bis(tetrahydroisoquinoline) framework have been found from other microorganisms[5a] and also from a marine sponge.[5b] Et's seem to share the biosynthetic origin of their

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(2) G. Faircloth, PharmaMar U.S., Cambridge, MA, personal communication to K. L. R.

(3) (a) Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* **1990**, *55*, 4512−4515. (b) Wright, A. E.; Forleo, D. A.; Gunawardana, P. G.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. *J. Org. Chem.* **1990**, *55*, 4508−4512. (c) Guan, Y.; Sakai, R.; Rinehart, K. L.; Wang, A. H.-J. *J. Biomol. Struc. Dynamics* **1993**, *10*, 793−818. (d) Sakai, R., Ph.D. Thesis, University of Illinois, Urbana, 1991.

(4) (a) Arai, T.; Takahashi, K.; Kubo, A. *J. Antibiot.* **1977**, *30*, 1015−1018. (b) Ikeda, Y.; Idemoto, H.; Hirayama, F.; Yamamoto, K.; Iwao, K.; Asao, T.; Munakata, T. *J. Antibiot.* **1983**, *36*, 1279−1283.

(5) (a) Remers, W. A. *The Chemistry of Antitumor Antibiotics*; Wiley: New York, 1988; Vol 2, pp 93−119. (b) He, H.-Y.; Faulkner, D. J. *J. Org. Chem.* **1989**, *54*, 5822−5824.

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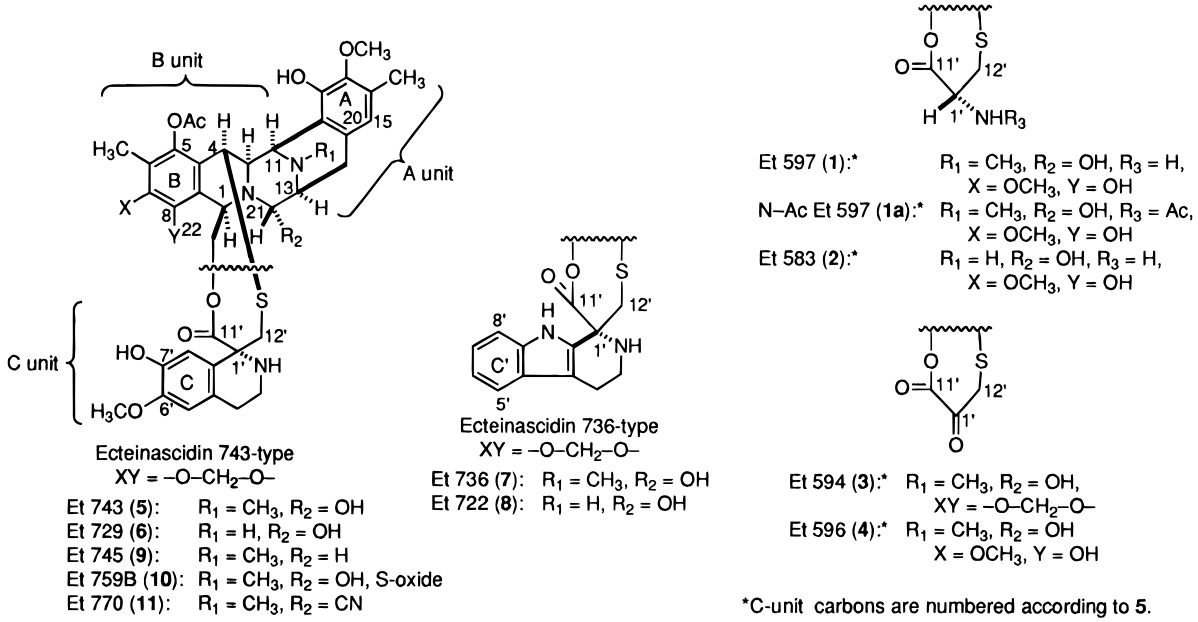
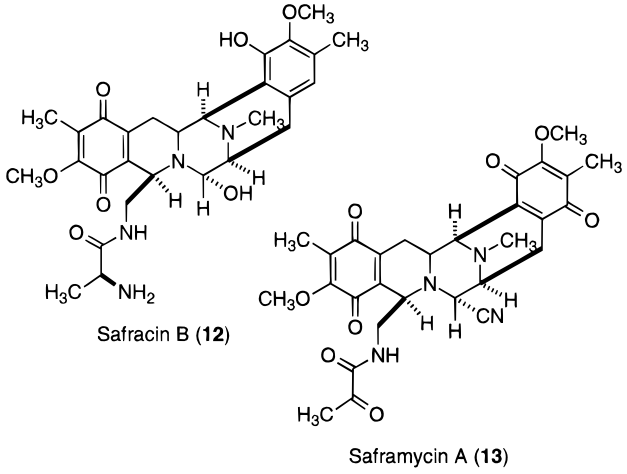
Chart 1



A−B unit with those saframycin-class compounds, especially with safracin B (**12**)[4b] for unit A. However, the third tetrahydroisoquinoline or the tetrahydro-β-carboline unit C attached to unit B by the 10-membered sulfide-containing lactone is a quite distinctive feature of the Et molecules, both structurally and biosynthetically. The potent anticancer activity of Et's may, at least in part, be attributed to the unit C, since the related saframycin A (**13**), for example, which lacks the C unit, has lower efficacy than Et 729 in comparable tumor models.[5a,6] Thus, it is likely that unit C in Et molecules has not only chemical but also biological importance:



We earlier proposed a biosynthetic scheme for the formation of the A−B and C units of Et's 729 and 743[1c,3a] which was later modified to include the new series of Et's 722 (**7**) and 736 (**8**) with a tetrahydro-β-carboline instead of a tetrahydroisoquinoline as unit C.[1g] Tryptamine was also isolated from the same source.[1g] This generalized our previously proposed biosynthetic scheme for the formation of unit C in which it would be formed by a Pictet−Spengler condensation of the cysteine-derived β-mercaptopyruvic acid and an aromatic amine to give a tetrahydroisoquinoline or a tetrahydro-β-carboline C unit which then adds to the A−B unit.

In the present study we report the isolation, structure assignments, and biological activities of the new ecteinascidins **1**−**4** (Et's 597, 583, 594, and 596), putative biosynthetic precursors of the Et's. Isolation of these compounds allows us to derive a more detailed scheme for the biogenesis of unit C which differs from that originally proposed.[1c,3a] Moreover, the absolute configuration of **1** has been determined unambiguously, allowing us to assign that for the medicinally important Et 743 (**5**).

Ecteinascidins 597 and 583 (1 and 2). New Et's **1**−**4** were isolated as described in the Experimental Section,[3d] and their molecular formulas are based on positive ion HRFABMS data on dehydrated "molecular" ions (M + H−H_2O).[1c,g;3a,b,d;8] NMR spectra of **1**, interpreted by COSY(correlation spectroscopy), HMBC (^{1}H-detected multiple bond heteronuclear multiple-quantum coherence), and HMQC (^{1}H-detected heteronuclear multiple-quantum coherence) data and those of **2** and **3**, assigned by COSY, HMQC, and analogy to other previously reported Et's[1c,g;3a,b,d] (Table 2), revealed a lack of aromatic signals for unit C, i.e., neither a third tetrahydroisoquinoline unit nor a tetrahydro-β-carboline unit. On the other hand, resonances assigned for the A−B unit in **1** and **2** were very similar to those of **5** except for lack of a methylenedioxy group (^{13}C NMR *ca.* 103 ppm; ^{1}H NMR *ca.* 6.1 ppm, AB quartet, J = *ca.* 1 Hz)[1g] in **1** and **2**. We have previously assigned the fragmentation patterns for Et's in FAB mass spectrometry using FABMS/CID/MS and high-resolution FAB mass spectra.[1c,g;3a,b] This detailed assignment of FABMS fragments for Et's is useful in identifying related biosynthetic precursors. FABMS/CID/MS data on M + H − H_2O of **1** (*m*/*z* 598) gave product ions a and b common to the A unit of **5** at *m*/*z* 204 and 218, but product ions f and g for unit B, at *m*/*z* 262 and 248, and c and d for the subunit A−B in **1**, at *m*/*z* 465 and 495, were shifted 2 Da higher from those of **5** (Scheme 1, Table 3).[1c] These MS observations and the NMR data suggested that unit A of **1** is the same as that of **5** and **7**, while unit B of **1** contains two more hydrogens, in agreement with replacement of the methylenedioxy group in

(6) Saframycin A exhibited antitumor activity against B16 melanoma, *T*/*C* = 146 (1 mg/kg, 1−5 days ip), and safracin B had *T*/*C* 200 (0.5 mg/kg, 1−9 days, ip),[5a] while Et 729 had *T*/*C* = 253 (12.5 μg/kg, 1−9 days, ip) with five survivors at day 42.[3d]

(7) The present compounds (**1**−**4**) have been briefly described in a meeting report: Rinehart, K. L.; Tachibana, K. *J. Nat. Prod.* **1995**, *58*, 344−358.

(8) Due to the reactive carbinolamine at C-21, compounds **1** and **2** gave only dehydrated molecular ions in positive FABMS spectra. Attempts to observe [M − H]$^-$ by negative FABMS failed (see ref 1e).

Table 2. ^{1}H and ^{13}C NMR Data for Et 743 (**5**) in $CD_3OD-CDCl_3$ (3:1) and 597 (**1**), 583 (**2**), and 594 (**3**) in CD_3OD

	chemical shift (δ), multiplicity[a] (J in Hz)							
	5		**1**		**2**[e]		**3**	
atoms[b]	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	56.3, d	4.78, br s	57.2, d	4.82, br s	58.2, d	4.73, br s	57.0, d	4.78, br s
3	58.8, d	3.72[c]	58.9, d	3.51, br d (3.5)	58.5, d	3.47, br d (5.0)	59.5, d	3.58, d (4.5)
4	42.7, d	4.58, br s	43.1, d	4.51, br s	nd	4.50, br s	42.5, d	4.45, br s
5	142.2, s		140.3, s		nd		nd	
6	113.9, s		124.3, s		nd		nd	
7	146.5, s[d]		146.4, s		nd		nd	
8	141.9, s		144.7, s		nd		nd	
9	116.0, s		115.6, s		nd		nd	
10	122.0, s		122.1 s		nd		nd	
11	55.6, d	4.40, br d (3.5)	56.0, d	4.22, br d, (4.0)	48.8, d	4.43, d (4.5)	56.5, d	4.21, m
13	54.0, d	3.52, br s	54.1, d	3.37, br m	47.2, d	3.63, br dd (8.5, 2.5)	55.1, d	3.38, m
14	24.5, t	2.91, 2 H, br d (4.5)	24.6, t	2.82, d, (5.0)	28.1, t	2.98, dd (17.5, 9.5) 3.07, d (17.5)	24.9, t	2.81, dd (17.0, 9.0) 2.69 d (17.0)
15	120.9, d	6.55, s	121.2, d	6.45, s	122.1, d	6.49, s	121.7 d	6.43, s
16	131.2, s		130.9, s		nd		nd	
17	145.1, s		145.7, s		nd		nd	
18	149.8, s		150.3, s		nd		nd	
19	119.2, s		120.3, s		nd		nd	
20	131.5, s		132.1, s		nd		nd	
21	92.1, d	4.26, d (3.0)	93.1, d	4.19, d (3.0)	91.5, d	4.15, d (2.5)	91.7, d	4.21, m
22	61.2, t	5.14, d (11.0) 4.09, dd (11.0, 2.0)	61.4, t	5.14, d (11.0) 4.31, dd (2.0, 11.0)	62.1, t	5.14, d (11.0) 4.32, dd (11.0, 2.0)	62.3, t	5.16, d (11.5) 4.08, dd (11.5, 2.5)
OCH_2O	103.1, t	6.07, d (1.0) 5.98, d (1.0)					103.6, t	6.11, d (1.0) 6.00, d (1.0)
1′	65.3, s		54.3, d	3.22, br m	54.9, d	3.22, br m	100.5, s	
3′	40.3, t	3.13, dt (11.0, 4.0) 2.77, ddd (3.5, 5.5, 11.0)						
4′	28.6, t	2.60, ddd (5.5, 10.5, 16.0) 2.42, ddd (3.5, 3.5, 16.0)						
5′	115.6, d	6.38, s						
6′	146.4, s[d]							
7′	146.4, s[d]							
8′	111.3, d	6.42, br s						
9′	125.4, s							
10′	128.8, s							
11′	173.1, s		174.8, s		nd[g]		nd[g]	
12′	43.1, t	2.38, br d (15.5) 2.05[c]	35.4, t	2.2, br	35.5, t	2.2, br	38.7, t	1.84, d (15.0)[f]
5-C=O	169.8, s		167.5, s					
5-OAc	20.5, q	2.29, s	20.8, q	2.29, s	21.2, q	2.29, s	20.4, q	2.29, s
6-CH_3	9.9, q	2.01, s	10.1, q	2.04, s	10.4, q	2.03, s	9.6, q	1.99, s
7-OCH_3			61.1, q	3.71, s	61.4, q	3.70, s		
16-CH_3	16.1, q	2.28, s	15.9, q	2.24, s	15.9, q	2.23, s	16.0 q	2.23, s
17-OCH_3	60.2, q	3.72, s	60.2, q	3.72, s	60.3, q	3.72, s	60.3, q	3.72, s
7′-OCH_3	55.7, q	3.58, s						
12-NCH_3	41.1, q	2.23, s	41.2, q	2.01, s			40.8, q	2.06, s

[a] s = singlet, d = doublet, t = triplet, q = quartet, br = broad. [b] Proton assignments are based on COSY and homonuclear decoupling experiments; carbon multiplicities were determined on the basis of either APT, DEPT, or HMQC data. [c] The signal overlaps the methyl singlet. [d] Assignments are interchangeable. [e] Carbon resonances were observed through proton resonances by HMQC experiment due to the limited amount of sample available. [f] The coupled proton is missing. [g] Not detected.

unit B of **5** and **7** by a methoxyl (^{1}H NMR 3.71, ^{13}C NMR 61.1 ppm) plus a hydroxyl group in **1** instead. The position of the 7-methoxyl group was confirmed by ROESY data (supporting information, Figure 5S) for $N^{1'}$-acetyl-Et 597 (**1a**), which showed NOE cross peaks, indicating that aromatic methyl and methoxyl groups are adjacent to each other in both units A and B (Scheme 2).

In addition to the A−B unit of **1** ($C_{27}H_{32}N_2O_8$), the rest of the molecule (C unit) consists of C_3H_5NOS, which contains two degrees of unsaturation, including an ester carbonyl at 174.1 ppm, linking unit C to unit B. COSY and HMBC data for **1** showed that the spin system for $-CHCH_2OCO-$, observed in all other Et's for C-1, C-22, and the ester carbonyl of unit C, respectively, is also present in **1**. HMQC data showed a broad singlet CHN proton at 3.22 ppm and carbon at 54.3 ppm. The proton shifted to 4.53 ppm on acetylation of **1** and correlated to an exchangeable NH proton at 5.48 ppm in **1a**, confirming a primary amine in **1**. A sulfur attached to C-4 in **1** was identified by NMR resonances for H-4 (4.51 ppm) and C-4 (43.1 ppm), very similar to those in other Et's (cf. **5** and **7**).[1c,g;3a,b] A methylene carbon at 35.4 ppm and two very broad protons at 2.20 ppm were also assignable to a sulfide-substituted $-CH_2-$.[1c-f] Although correlation spectra (COSY, HMBC) failed to connect this broad sulfide methylene[9] with the CHN proton α to the ester carbonyl, due to the small sample size and broad, weak signals, these two groups must be connected to form a 10-membered sulfide-containing lactone as in other Et's. The relationship was also assured by the isolation of cysteine itself (cf. below). Thus, the structure of **1** was assigned as depicted in Chart 1.[10]

(9) Signal broadening in medium-sized rings has been commonly observed for marine polyethers; *cf.* Murata, M.; Legrand, A. M.; Ishibashi, Y.; Yasumoto, T. *J. Am. Chem. Soc.* **1989**, *111*, 8929−8931.

(10) ROESY data confirmed all other stereochemistry of the A−B unit of **1** to be the same as in **5**; see Scheme 2. X-ray data for derivatives of **5** had revealed all relative stereochemistry for the A−B unit.[1f]

Scheme 1

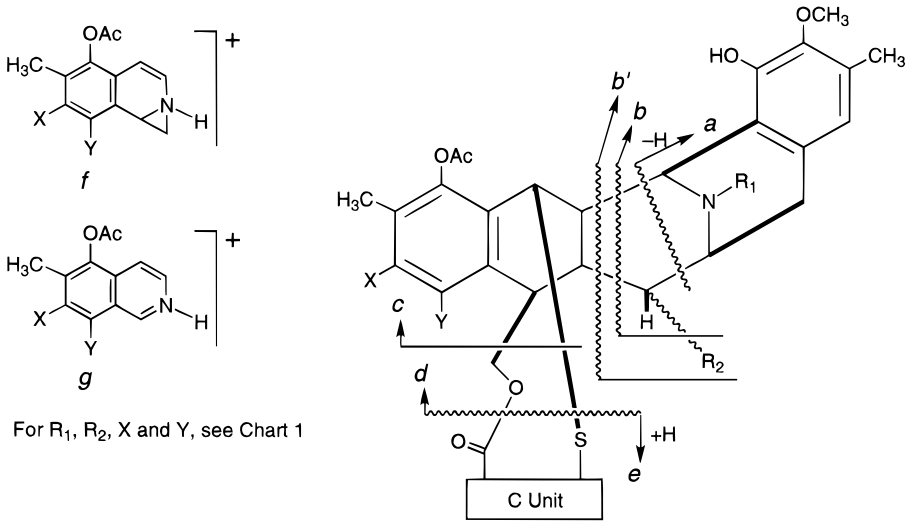
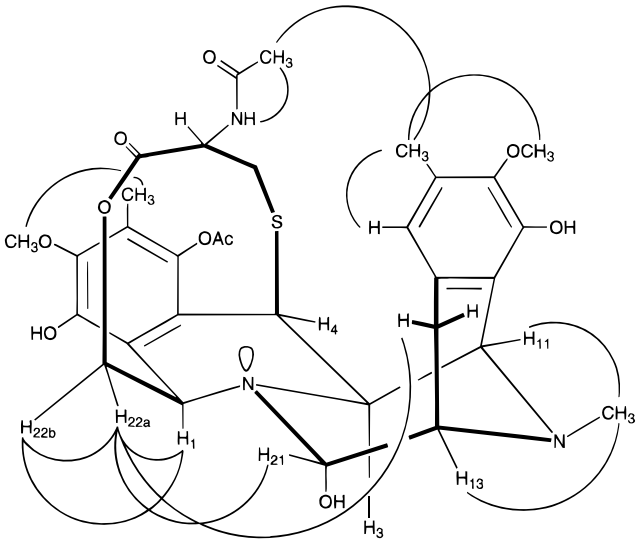


Table 3. FABMS Data for Ecteinascidins (see Scheme 1)

				product ions (MS/CID/MS or HRFABMS)						
compound	formula	molecular ion	M−H	a	b	c	d	e	f	g
Et 743 (**5**)[a]	$C_{39}H_{43}N_3O_{11}S$	$C_{39}H_{42}N_3O_{10}S$ 744.2591 Δ 5.7	$C_{39}H_{42}N_3O_{11}S$ 760.2514 Δ 2.6	$C_{12}H_{14}NO_2$ 204.1027	$C_{13}H_{16}NO_2$ 218.1174	$C_{26}H_{27}N_2O_6$ 463.1862	$C_{27}H_{29}N_2O_7$ 493.1980	$C_{11}H_{14}NO_2S$ 224.0749	$C_{14}H_{14}NO_4$ 260.0911	$C_{13}H_{12}NO_4$ 246.0757
Et 729 (**6**)[a]	$C_{38}H_{41}N_3O_{11}S$	$C_{38}H_{40}N_3O_{10}S$ 730.2493 Δ −5.0	$C_{38}H_{40}N_3O_{11}S$ 746.2376 Δ 0.8	$C_{11}H_{12}NO_2$ 190	$C_{12}H_{14}NO_2$ 204	$C_{25}H_{25}N_2O_6$ 449	$C_{26}H_{27}N_2O_7$ 479	224	260	246
Et 745 (**9**)[a]	$C_{39}H_{43}N_3O_{10}S$	$C_{39}H_{44}N_3O_{10}S$ 746.2775 Δ −2.8	NO[d]	204	218	463	493	224	260	246
Et 597 (**1**)	$C_{30}H_{37}N_3O_9S$	$C_{30}H_{36}N_3O_8S$ 598.2219 Δ 0.4	NO	204	218	465	495	NO	262 (s)[e]	248
Et 583 (**2**)	$C_{29}H_{35}N_3O_9S$	$C_{29}H_{34}N_3O_8S$ 584.2054 Δ 1.2	NO	190	204	451	481	NO	262 (s)	248
Et 594 (**3**)[b]	$C_{30}H_{32}N_2O_{10}S$	$C_{30}H_{31}N_2O_9S$ 595.1716 Δ 3.4	NO	204	218	463	493	NO	NO	NO
Et 596 (**4**)	$C_{30}H_{34}N_2O_{10}S$	$C_{31}H_{37}N_2O_{10}S$[c] 629.2171 Δ −0.3	NO	204	218	465	495	NO	262 (s)	248

[a] Data taken from ref 1c,e. [b] MS/MS of *m*/*z* 627 product (M + MeOH). [c] Methanol adduct. [d] NO = not observed. [e] (s) = small peak.

Scheme 2

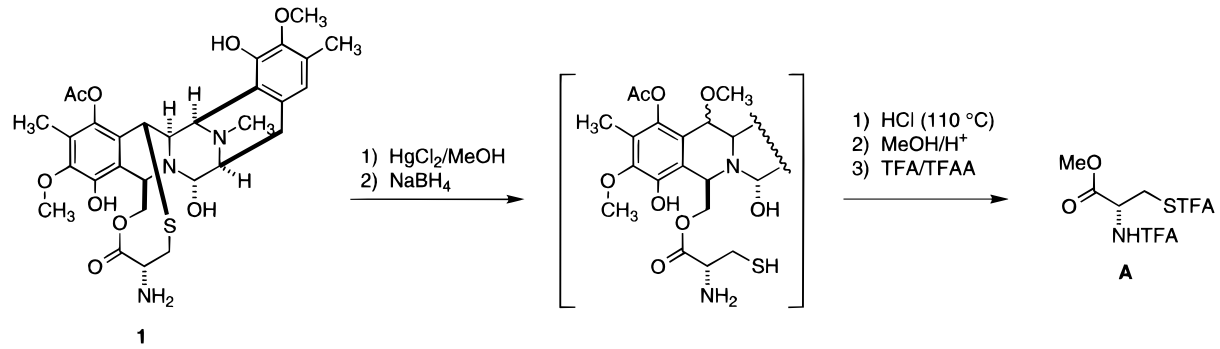


1a

Ecteinascidin 583 (**2**) is the N^{12}-demethyl analog of **1**. In the ^{1}H NMR spectrum of **2** only three methyl groups are observed in the region from 2.0 to 2.5 ppm, whereas four methyl signals appeared in the spectrum of **1**. The molecular formula of **2**, $C_{29}H_{35}N_3O_9S$, also corresponds to the lack of a methyl. FABMS/CID/MS spectra of **2** gave product ions a and b at *m*/*z* 190 and 204, 14 Da less than those from **1** (Table 3). COSY and HMQC data for **2** were compared with other ecteinascidins' NMR data, allowing assignment of all the protons and protonated carbons. Protonated carbons (Table 2) C-11 and C-13 were shifted upfield compared to those of **1** as a result of the demethylation at N-12, while ^{1}H NMR signals were shifted downfield. These shifts in the NMR data were previously observed between the N^{12}-methyl and N^{12}-demethyl analogs of Et's.[1c,g;3a,b,d] All the above data are consistent with the assignment of ecteinascidin 583 (**2**) as the N^{12}-demethyl analog of Et 597 (**1**), just as Et 729 (**5**) and Et 722 (**7**) are N^{12}-demethyl analogs of Et 743 (**6**) and Et 736 (**8**).[1g]

Absolute Stereochemistry. A ROESY NMR spectrum of **1a** showed an NOE between the amide proton and the methyl protons of the *N*-acetyl group, and an NOE between the *N*-acetyl methyl group and the 16-methyl group of unit A revealed the relative stereochemistry at C-1′ as in Scheme 2. Treatment of **1** with $HgCl_2$ followed by $NaBH_4$ and methanolysis gave a mixture containing cysteine methyl ester. This product was derivatized with trifluoroacetic anhydride (TFAA) and trifluoroacetic acid (TFA) and then analyzed by chiral GC (Scheme 3). Injection of the analyte with D,L-TFA-Cys-OMe showed that the derivatized Cys (**A**) in the mixture coeluted with the L-isomer of the derivatized racemate (Figure 1). Thus, the absolute stereochemistry of **1** at C-1′ was determined to be *R*. Since the relative stereochemistry of the C unit and that of the A−B unit was related by the above ROESY experiment, the stereochemistry of **1** is assigned as 1*R*,2*R*,3*R*,4*R*,11*R*,13*S*,-21*S*,1′*R*. This agrees with that presumed from the X-ray study but differs from that proposed initially for **5**.[3a,b]

Scheme 3



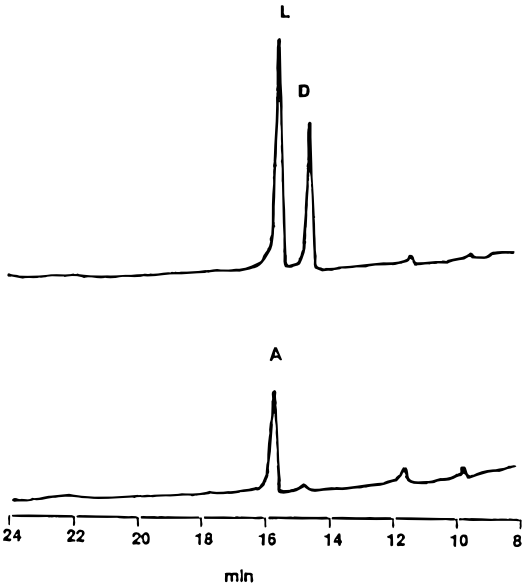


Figure 1. Gas chromatogram for: reaction product **A** (below) and coinjection of **A** and bis-TFA-D,L-Cys-OMe (above) on Chirasyl Val-III (isothermal, 100 °C).

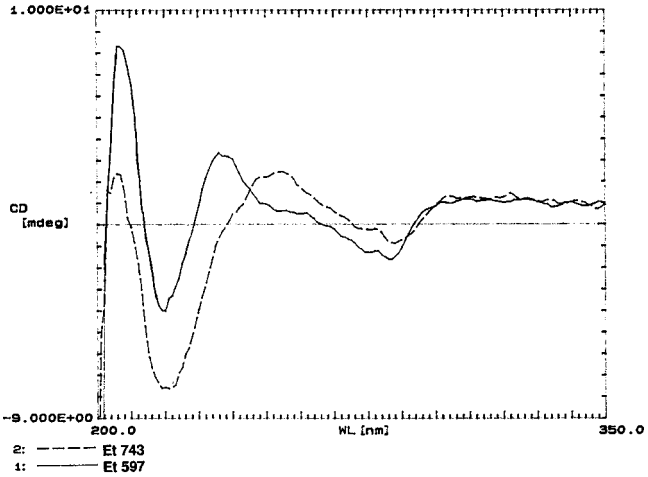


Figure 2. CD spectra for Et 597 (**1**) (solid) and Et 743 (**5**) (broken) in CH_3OH.

Et 743 (**5**) must have the same absolute stereochemistry, since the CD spectrum for **1** was very similar to that of **5** (Figure 2). Moreover, it is most plausible biosynthetically that all other Et's have the same absolute stereochemistry for the A−B unit's chiral centers.

Ecteinascidins 594 (3) and 596 (4). Compound **3** was obtained as a methanol adduct, giving a protonated "molecular" ion (M + H − H_2O + MeOH) at *m*/*z* 627 in the magic bullet matrix.[11] The FABMS spectra of the methanol adduct of **3** in glycerol matrix alone gave only the [M + H − H_2O + MeOH]$^+$ ion at *m*/*z* 627 (627.2020, HRFABMS); however, peaks at *m*/*z* 595.1750 (M + H − H_2O, relative intensity 27%), 613.1827 (M + H, 52%), and 687.2205 (M + H − H_2O + glycerol, 60%) were observed when a small amount of aqueous oxalic acid was added. ^{13}C and ^1H NMR data showed that the A−B unit of **3** is very similar to that of **5**, and a FABMS/CID/MS spectrum of the methanol adduct (*m*/*z* 627) gave product ions a−d at *m*/*z* 204, 218, 463, and 493, respectively, fragments common to the A−B unit in **5**. If the subunit A−B of **3** is the same as that of **5**, the adducted methanol must be located on unit C. The C unit of **3** consists of $C_3H_2O_2S$ (three double bond equivalents) which is assigned to be a 2-oxo-2-deaminocysteine unit, as depicted in Chart 1. The ^{13}C NMR spectrum for **3** in CD_3OD showed a signal at 100.5 ppm assignable to a hemiketal at C-1′. An IR spectrum (film) of **3**, with a shoulder at 1734 cm^{-1} as opposed to a sharp single absorption for esters as seen in **1**, supported the presence of an α-keto ester group in **3**.[12]

Et 596 (**4**) was observed only in a mixture as a methanol adduct; it gave a protonated "molecular" ion at *m*/*z* 629.2171 ($C_{31}H_{37}N_2O_{10}S$), which corresponds to that of **3** with two more hydrogens. This could either be due to the replacement of the methylenedioxy group by OCH_3 and OH groups in the B unit as in **1** or to the presence of the C-21 reduced structure seen in Et 745 (**9**), which usually gives a true M + H ion.[1c,3a] An attempt to obtain NMR data for this compound failed due to the minute amount of **4** in the mixture, but FABMS/CID/MS of the *m*/*z* 629 (M + H − H_2O + MeOH) parent gave daughter ions a and b at *m*/*z* 204 and 218 for unit A, and ions f and g at *m*/*z* 465 and 495 for unit A−B, respectively (Table 3), suggesting that **4** has the intact A unit of Et 743 and the B unit plus H_2, as seen in **1**. Addition of excess cyanide to a methanolic solution of the mixture followed by a FABMS measurement gave ions for a monocyano (at *m*/*z* 624) and a dicyano adduct of **4** at *m*/*z* 651 (Figure 3), indicating the presence of two electrophilic centers, the C-21 carbinolamine and the C-1′ oxo groups. Although these results are tentative, they are consistent with the structure of Et 596 as **4**, a C-1′-deamino, C-1′-oxo derivative of **1**.

Biogenesis of the Ecteinascidins (Scheme 4). As proposed earlier,[1c,3a] A−B units of Et's are most likely formed by condensation of two Dopa-derived building blocks, and the tetrahydroisoquinoline ring in unit B is closed by condensation (Pictet-Spengler) with a serine- (or glycine-) derived aldehyde as in the case of the related saframycins.[13] *S*-Adenosylmethionine is the likely source of methyl groups at C-6, O-7, C-16,

(11) Magic bullet contains 10% methanol; *cf*. Witten, J. L.; Schaffer, M. H.; O'Shea, M.; Cook, J. C.; Hemling, M. E.; Rinehart, K. L., Jr. *Biochem. Biophys. Res. Commun.* **1984**, *106*, 811−813.

(12) IR absorption for the α-keto group is expected to be small, since compound **1** exists mostly as the hemiketal or ketal in solution, as evidenced by FABMS. In the solid form, it may also exist, at least in part, as the hemiketal or ketal form.

(13) Mikami, Y.; Takahashi, K.; Yazawa, K.; Arai, T.; Namikoshi, M.; Iwasaki, S.; Okuda, S. *Biol. Chem.* **1985**, *260*, 344−348.

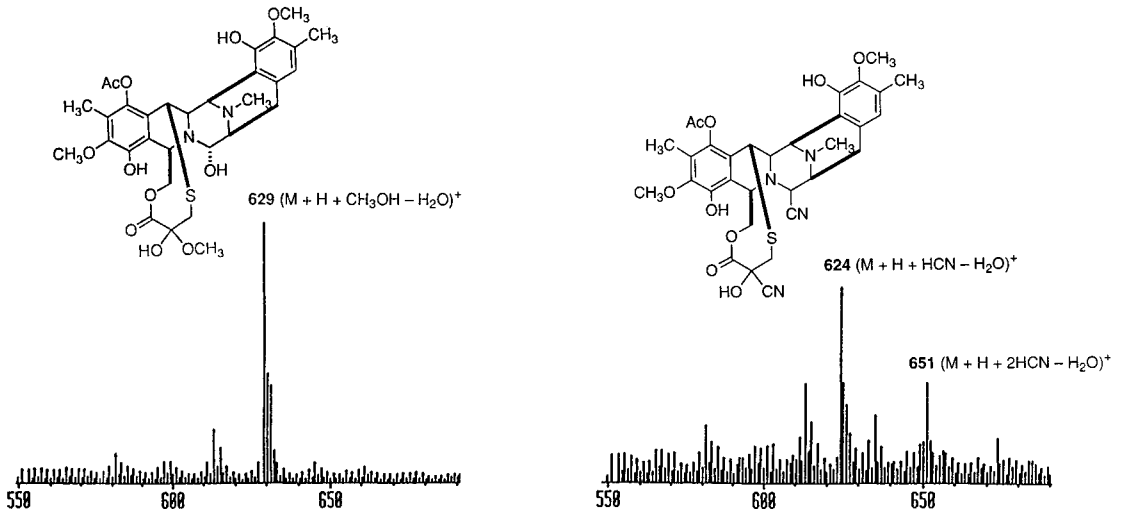
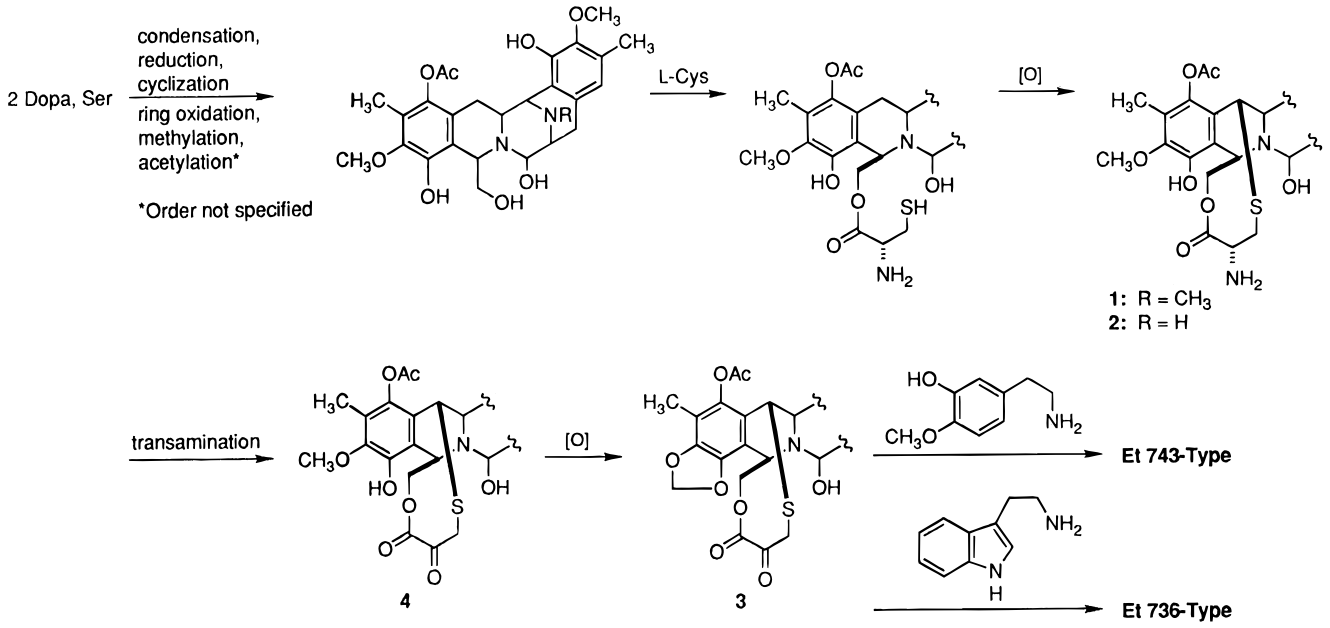


Figure 3. FAB mass spectrum for Et 596 (**4**) in the presence of CH_3OH (*m*/*z* 629, M + H + CH_3OH − H_2O)$^+$ and in the presence of CN^- (*m*/*z* 624, M + H + HCN − H_2O; *m*/*z* 651, M + H + 2HCN − H_2O).

Scheme 4



O-17, and N-12. The scheme has been partially confirmed by incorporation of labeled tyrosine,[14] cysteine,[14] methionine,[14b] glycine,[14b] and tryptophan.[14b] The sequence of the oxidation, methylation, and acetylation at the aromatic rings is not yet clear.

The present study offers the most plausible route for the formatiuon of unit C to be that in Scheme 4, with the side chain alcohol at C-22 being acylated by cysteine, followed by oxidation and addition (or substitution) at C-4 by the SH group of the side chain with closure of the 10-membered lactone ring to give **1**. Transamination at C-1′ forms an oxo derivative, **4**, then oxidation of the 7-OCH_3 of **4** yields the methylenedioxy group to form **3**.[15] The electrophilic ketone in **3** can be condensed in a Pictet−Spengler reaction with a dopamine derivative to form the third tetrahydroisoquinoline group in Et 743 (**5**) or with tryptamine to form the tetrahydro-β-carboline group in Et 736 (**7**).[1g] A similar biogenetic origin can be proposed for Et's 729 (**6**) and 722 (**8**), involving Et 583 (**2**) as an intermediate. This scheme differs from that suggested earlier[1c,3a] in which the C unit was formed independently by condensation of *O*-methyldopamine or tryptamine with mercaptopyruvate, then coupled to unit A−B. Biosynthetic studies to confirm or modify the present scheme are in progress.[14]

Bioactivities of New Et's. Biological activities of new Et's **1**−**3**, Et 743 (**5**), and Et 729 (**6**), including cytotoxicity against several cell lines, antimetabolic activities, enzyme inhibitions and antimicrobial activity, are summarized in Table 4. New Et's which lack the aromatic C unit were generally 10−50 times less active than **5** and **6**, except for **1**, against the MEL 28 and CV-1 cell lines. All tested Et's showed potent inhibition of DNA and RNA synthesis and of RNA polymerase activity, but much less inhibition of DNA polymerase activity. Previously we proposed a possible binding model of Et 729 (**6**) and a DNA oligomer since it had been shown to bind DNA.[1g,16] In this study, however, it is shown that Et's possess a stronger inhibitory effect against RNA synthesis and RNA polymerase activity relative to DNA. Saframycin A (**13**) was also shown to inhibit RNA and DNA synthesis but not protein synthesis

(14) (a) Kerr, R. G.; Miranda, N. F. *J. Nat. Prod.* **1995**, *58*, 1616−1621. (b) Morales, J. J.; Rinehart, K. L. 1995 International Chemistry Congress Pacific Basin Societies, Honolulu, Dec 17−22, 1995, Abstract ORGN 184.

(15) Enzymatic reactions to form a methylenedioxy bridge have been demonstrated for plant-derived tetrahydroisoquinolines; *cf*. Bauer, W.; Zenk, M. H. *Phytochemistry* **1991**, *30*, 2953−2961.

(16) Guan, Y.; Sakai, R.; Rinehart, K. L.; Wang, A. H.-J. *J. Biomol. Struct. Dyn.* **1993**, *10*, 793−818.

Table 4. Cytotoxicity,[a] Antimetabolism,[b] Enzyme Inhibition,[c] and Antimicrobial Activity[d] of Et's

	cell lines, IC_{50} (ng/mL)					synthesis inhibition, IC_{50} (μg/mL)			enzyme inhibition, IC_{50} (μg/mL)		*B. sub.*,[d]
compounds	P388[a]	A549[a]	HT29[a]	MEL28[a]	CV-1[a]	prot[b]	DNA	RNA	DNAp[c]	RNAp[c]	MIC (μg/disk)
Et 743 (**5**)	0.2	0.2	0.5	5.0	1.0	>1	0.1	0.03	2	0.1	0.02
Et 729 (**6**)	0.2	0.2	0.5	5.0	2.5	>1	0.2	0.02	1.5	0.05	0.08
Et 597 (**1**)	2.0	2.0	2.0	2.0	2.5	0.7	0.08	0.01	−	0.25	0.14
Et 583 (**2**)	10	10	10	5.0	25	1.0	1.0	0.4	−	0.5	0.74
Et 594 (**3**)	10	20	25	25	25	0.8	0.5	0.5	−	1.0	0.37

[a] P388 = murine lymphoma; A549 = human lung carcinoma; HT29 = human colon carcinoma; MEL28 = human melanoma; CV-1 = monkey kidney. [b] prot = protein synthesis. [c] DNAp = DNA polymerase inhibition; RNAp = RNA polymerase inhibition. [d] *Bacillus subtilis.*

following activation by dithiothreitol.[17] It is interesting to note that the new Et's showed some inhibition of protein synthesis, while **5** and **6** were less active. More detailed biochemical studies on these Et analogues will be necessary to specify the mechanism of action of the ecteinascidins.

Experimental Section

General. NMR spectra were obtained on a 500 MHz FT NMR spectrometer using either $CDCl_3$, CD_3OD, or a mixture of both as solvents [internal standards: δ 7.26 (^{1}H) and 77.0 (^{13}C) ppm for $CHCl_3$; 3.30 (^{1}H) and 49.0 (^{13}C) ppm for CD_3OD or a mixture of $CD_3OD-CDCl_3$]. FABMS, FABMS/CID/MS, and HRFABMS data were recorded on a VG 70-SE-4F spectrometer. Optical rotations were measured with a JASCO DIP 370 digital polarimeter with a Na lamp (589 nm) using a 5 cm × 0.35 cm (1.0 mL) cell. CD spectra were obtained on a JASCO J720 spectropolarimeter. Reversed phase HPLC was performed using a C-18 semipreparative column (Altex, 10 μm gel, 10 × 250 mm), a UV detector (254 nm), and a flow rate of 1 mL/min. An Ito coil (P.C. Inc.) was used for high-speed countercurrent chromatography (HSCCC) at 800 rpm.

Separation. A specimen of *E. turbinata*, collected in Puerto Rico in September 1992, was stored frozen until use. A frozen sample (2.8 kg) was extracted twice with 2-propanol (4 L each time, at less than 5 °C) for 10 h. The alcoholic extract was evaporated to an aqueous emulsion (2.5 L) which was extracted with EtOAc (1 L × 1, 0.5 L × 1). The organic layer was concentrated and then partitioned between the lower and upper layers of a solvent system of EtOAc−heptane−MeOH−H_2O, 7:4:4:3 (200 mL). The upper and lower layer gave 1.03 and 0.226 g of solids, respectively, upon concentration. The solid obtained from the lower layer was separated by C18 (25 g) flash chromatography. The first eluent (MeOH−0.4 M aqueous NaCl, 9:2, 50 mL) afforded bioactive fraction A (89.3 mg), and the second fraction (MeOH−$CHCl_3$ wash) gave mostly lipids (116.5 mg). Fraction A was flash chromatographed over silica gel (pretreated with NH_3, 0.5% w/w). The first (1:9 MeOH−$CHCl_3$ eluate, fraction B) and second (1:4 MeOH−$CHCl_3$ eluate) fractions exhibited activity against *Bacillus subtilis* (12 mm zone at 0.3 μg/disk).

A stored (−20 °C) specimen (50 kg) collected during 1992 and 1993 was processed similarly, giving fraction B′.

Fraction B′ (80 mg) was separated by high-speed countercurrent chromatography (HSCCC, solvent toluene−Et_2O−MeOH−H_2O, 6:6:6:3; lower layer mobile phase; flow rate 1.8 mL/min) into 9 fractions (fractions C-1 to C-9). Fraction C-1 (9.9 mg) was separated by RPHPLC (MeOH−NaCl 0.02 M, 3:1) to give mainly four fractions. The first and second fractions were combined and then separated on a silica gel column (1.5 × 25 cm column, $CHCl_3$−MeOH, 6:1) to give pure Et 597 (**1**, 1.45 mg) and Et 583 (**2**, 1.43 mg).

Ecteinascidin 597 (1): Light brown solid; $[\alpha]^{25}_D$ −49° (*c* 0.17, MeOH); IR (film) 3400, 2934, 1741, 1456, 1419, 1236, 1197, 1066, 995, 754 cm^{-1}; UV (λ_{max}) 207 (ϵ 46 000), 230 (sh, 15 000), 278 (3500), 285 (3800); CD (λ_{ext}) 207 (ΔE 15.4), 219 (−7.4), 237 (6.5), 280 (−2.8), 287 (−3.3). For NMR data, see Table 2. Anal. calcd for $C_{30}H_{36}N_3O_8S$ (M + H − H_2O) M_r 598.2223, found M_r 598.2219 (HRFABMS).

Et 583 (2): Light yellow solid; $[\alpha]^{22}_D$ −47° (*c* 0.14, $CHCl_3$−MeOH, 6:1); UV (λ_{max}) 207 (ϵ 48 000), 230 (sh, 9200), 280 (2100), 290 (2300). For NMR data, see Table 2. Anal. calcd for $C_{30}H_{36}N_3O_8S$ (M + H − H_2O) M_r 598.2223, found M_r 598.2219 (HRFABMS).

Fraction C-8 was purified by RPHPLC (same conditions as above). A broad peak (t_R = 33−42 min) gave Et 594 (**3**, 1.2 mg).

Et 594 (3): Light yellow solid; $[\alpha]^{22}_D$ −58° (*c* 1.1, MeOH); UV (λ_{max}) 207 (ϵ 60 500), 230 (sh, 11 000), 287 (2900); FABMS *m*/*z* 627 (M + H + MeOH − H_2O, magic bullet matrix), 595 (M + H − H_2O), 613 (M + H), 687 (M + H + glycerol − H_2O, glycerol matrix in presence of oxalic acid and water). For NMR data, see Table 2. Anal. calcd for $C_{30}H_{31}N_2O_9S$ (M + H − H_2O) M_r 595.1750, found M_r 595.1716 (HRFABMS).

Fraction C-6 was separated by HPLC (MeOH−NaCl 0.04 M, 3:1) to yield a fraction (0.5 mg) which showed a FABMS peak at *m*/*z* 629 for compound **4** as the major Et component.

***N*-Acetyl Et 597 (1a):** Et 597 (1 mg) was treated with Ac_2O (50 μL) and Et_3N (5 μL) at room temperature for 30 min. The product was passed through a Sep-pak silica gel column with $CHCl_3$−MeOH (9:1) and then purified by RPHPLC (9:2 MeOH−NaCl, 0.04 M) to give a monoacetyl derivative (0.5 mg): FABMS *m*/*z* 641 (M + H − H_2O); ^{1}H NMR ($CDCl_3$) δ 6.70 (1H, s), 5.48 (1H, brm), 5.12 (1H, d, J = 12.0 Hz), 5.10 (1H, brs), 4.87 (1H, brs), 4.53 (1H, m), 4.32 (1H, dd, J = 11.5, 2 Hz), 4.22 (1H, brd, J = 2.5 Hz), 4.00 (1H, brd, J = 8.5 Hz), 3.82 (3H, s), 3.80 (3H, s), 3.47 (1H, d, J = 18.5 Hz), 3.10 (1H, dd, J = 18.5, 8.5 Hz), 2.58 (3H, s), 2.36 (3H, s), 2.27 (3H, s), 2.08 (3H, s), 1.87 (3H, s), and a small amount of diacetyl derivative (only enough to obtain FABMS data). Anal. calcd for $C_{32}H_{39}N_3O_9S$ (M + H − H_2O) M_r 641.2407, found M_r 641.2398 (HRFABMS). Anal. calcd for $C_{34}H_{41}N_3O_{10}S$ (M + H − H_2O) M_r 683.2513, found M_r 683.2492 (HRFABMS).

Degradation of Ecteinascidin 597. GC Analysis. Compound **1** (0.5 mg) in methanol was treated with $HgCl_2$ (1 mg) followed by $NaBH_4$. A dark precipitate was removed by filtration and the mixture was treated with acidic methanol (MeOH−10% HCl, 110 °C, 30 min). The solvent was removed and the product was derivatized with trifluoroacetic anhydride (TFAA−TFA, 100 μL each, 110 °C, 15 min). The TFA derivative was then analyzed by chiral GC (Chirasil Val-III, 110 °C, isothermal). The standard sample of D,L-TFA-Cys-OMe was also prepared as above by methylation and TFA derivatization.

Acknowledgment. This paper is dedicated to our longtime colleague Professor Nelson Leonard, who is celebrating his 80th birthday. We thank Drs. D. Lednicer and S. Donohue, National Cancer Institute, for in vivo data. In vitro biological testing was performed by Drs. T. García de Queseda and D. García Grávalos, PharmaMar, S.A. This work was supported in part by research grants from the National Institute of Allergy and Infectious Diseases (AI01278, AI04769).

Supporting Information Available: ^{1}H NMR spectra of **1**−**3**, ^{13}C NMR spectrum of **1**, all in CD_3OD, and ROESY spectrum ($CDCl_3$) of **1a** (5 pages). See any current masthead page for ordering and Internet access instructions.

JA950054M

(17) Ishiguro, K.; Sakiyama, S.; Takahashi, K.; Arai, T. *Biochemistry* **1978**, *17*, 2545−2550.