Biosynthetic Studies of Marine Lipids. 26.[1] Elucidation of the Biosynthesis of Mutasterol, a Sponge Sterol with a Quaternary Carbon in Its Side Chain

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Mutasterol belongs to an unusual class of sterols that possess side chains with quaternary carbons. The biosynthesis of this sterol (1) in the Caribbean sponge *Xestospongia muta* has been elucidated by feeding of selected [3-^{3}H]sterol precursors. Codisterol (9) was ten times more efficiently transformed than its C_{24} epimer, while there was no difference in the degree of metabolism of the *E* and *Z* isomers of 24,26-dimethyldesmosterol (13, 14).

Introduction

Sponges are a rich source of unconventional sterols, many with no terrestrial counterparts.[2] In fact, over 200 new sterols have been found in marine organisms, most of them isolated from sponges. During the past few years major progress has been made in the elucidation of the biosynthesis of many of these unusual sterol side chains. This has included simple chain elongation,[3] double chain elongation,[4] hydrogen rearrangements in multiple bioalkylation,[5] order of methyl group introduction in multiply alkylated side chains,[6] and the mode of biosynthesis of the unique cyclopropane[7] and cyclopropene[8] moieties found in sponge sterols. This leaves one group of intriguing sterol side chains that has so far been encountered only in marine organisms and on occasion in trace quantities in certain plants:[9] sterol side chains with quaternary centers (Figure 1) such as mutasterol (1),[10] xestospongesterol (2),[11a] 25-methylxestosterol (3),[11b] and the sponge sterol 4.[11c]

Earlier we had proposed[2a,10] "paper" biosynthetic schemes for this important subclass of sterols and we now describe experimental verification of the earlier proposal by incorporation experiments with mutasterol (1)—a typical sterol of this subgroup. Mutasterol (1) was isolated[10] from the Caribbean sponge *Xestospongia muta*. The side chain is the product of a triple biomethylation and has a quaternary center at C_{25}. A tetracyclic triterpene (5) with the same side chain as mutasterol has been isolated[12] in trace quantities from a higher plant. Three hypothetical biosynthetic pathways were proposed[12a] for this triterpene.

Results and Discussion

Mutasterol (1) was first isolated[10] as a minor constituent of the sponge *Xestospongia muta* collected at Barbados. For logistical reasons, we decided to perform our biosynthetic experiments on *X. muta* from Puerto Rico. In the course of a biosynthetic project with a closely related sponge in Australia, *X. testudinaria*,[13] we found that there were two very different sterol compositions. Some specimens contained xestosterol (6), biosynthetically closely related to mutasterol (1), while others contained only conventional sterols.[14] We therefore analyzed the sterols of small fragments from nine individuals of *X. muta* from southwest Puerto Rico. Identification tags were attached to these sponges so that the individuals which contain mutasterol could be relocated for the biosynthetic studies. All of these sponges were from a small area, ca. 10 m × 10 m.

Quite unexpectedly, we found *three* different sterol compositions in these nine individuals (see Table I). As with *X. testudinaria* in Australia, one type (type A, found in three of the nine individuals sampled) possessed only conventional sterols and a second (type B, found in two of the nine samples) had xestosterol (6) as the predominant sterol. The third type (type C, found in four of the nine samples) was very similar to the sample of *X. muta* collected in Barbados, which contained mutasterol (1). It should be stressed that the three types were collected from sponge individuals within a small area and that the sterol compositions were not related to any apparent morphological character of the sponge.

In retrospect it is perhaps not surprising that two of the nine samples were found to contain xestosterol (6), as four years prior to our isolation of mutasterol from *X. muta*, Minale and co-workers[15] reported the isolation of xesto-

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(1) For part 25 in this series, see: Cordeiro, M. L.; Djerassi, C. *J. Org. Chem.* **1990**, *55*, 2806.

(2) (a) Djerassi, C. *Pure Appl. Chem.* **1981**, *53*, 873. (b) Ikekawa, I. *Sterols and Bile Acids*; Danielson, H., Sjovall, J., Eds.; Elsevier: Amsterdam, 1985; Chapter 8. (c) Withers, N. *Marine Natural Products*; Scheuer, P. J., Ed.; Academic Press: New York, 1983; Vol. 5, p 87. (d) Djerassi, C. *Alfred Benzon Symposium 20: Natural Products and Drug Development*; Krogsgaard-Larsen, P., Brøgger Christensen, S., Kofod, H., Eds.; Munksgaard: Copenhagen, 1984; p 164.

(3) Catalan, C. A. N.; Thompson, J. E.; Kokke, W. C. M. C.; Djerassi, C. *Tetrahedron* **1985**, *41*, 1073.

(4) Stoilov, I. L.; Thompson, J. E.; Djerassi, C. *Tetrahedron Lett.* **1986**, *27*, 4821.

(5) (a) Stoilov, I. L.; Thompson, J. E.; Djerassi, C. *Tetrahedron* **1986**, *42*, 4147. (b) Stoilov, I. L.; Back, T. G.; Thompson, J. E.; Djerassi, C. *Ibid.* **1986**, *42*, 4156.

(6) Stoilov, I. L.; Thompson, J. E.; Cho, J.-H.; Djerassi, C. *J. Am. Chem. Soc.* **1986**, *108*, 8235.

(7) (a) Proudfoot, J. R.; Catalan, C. A. N.; Djerassi, C.; Sica, D.; Sodano, G. *Tetrahedron Lett.* **1986**, *27*, 423. (b) Doss, G. A.; Proudfoot, J. R.; Silva, C. J.; Djerassi, C. *J. Am. Chem. Soc.* **1990**, *112*, 305.

(8) (a) Margot, C.; Catalan, C. A. N.; Proudfoot, J. R.; Sodano, G.; Sica, D.; Djerassi, C. *J. Chem. Soc., Chem. Commun.* **1987**, 1142. (b) Doss, G. A.; Margot, C.; Sodano, G.; Djerassi, C. *Tetrahedron Lett.* **1988**, *29*, 6051. (c) Doss, G. A.; Silva, C. J.; Djerassi, C. *Tetrahedron* **1989**, *45*, 1273.

(9) (a) Akihisa, T.; Yokota, T.; Takahashi, N.; Tamura, T.; Matsumoto, T. *Phytochemistry* **1989**, *28*, 1219. (b) Akihisa, T.; Tamura, T.; Matsumoto, T. *Ibid.* **1987**, *26*, 575.

(10) Li, L. N.; Sjöstrand, U.; Djerassi, C. *J. Am. Chem. Soc.* **1981**, *103*, 115.

(11) (a) Li, L. N.; Djerassi, C. *J. Am. Chem. Soc.* **1981**, *103*, 3606. (b) Li, L. N.; Sjöstrand, U.; Djerassi, C. *J. Org. Chem.* **1981**, *46*, 3867. (c) Shubino, L. K.; Makar'eva, T. N.; Kalinovski, A. I.; Stonik, V. A. *Khim. Prir. Soedin.* **1985**, *2*, 232.

(12) (a) Croft, J. A.; Ritchie, E.; Taylor, W. C. *Aust. J. Chem.* **1975**, *28*, 2019. (b) Nogai, M.; Nagumo, S.; Izawa, K. *Tetrahedron Lett.* **1975**, 3655.

(13) Zimmerman, M.; Djerassi, C. To be published.

(14) Conventional sterols are those in which the only alkylation is either a methyl or ethyl group at C_{24}; for more details, see: Nes, W. R.; McKean, M. L. *Biochemistry of Steroids and Other Isopentenoids*; University Park Press: Baltimore, 1977; Chapter 9.

(15) Minale, L.; Riccio, R.; Scalano, O.; Sodano, G.; Farrurosso, E.; Magno, S.; Mayol, L.; Santracroce, C. *Experientia* **1977**, *33*, 1550.

0022-3263/91/1956-0063$02.50/0

Table I. Sterols of Nine Individuals of *Xestospongia muta* from Puerto Rico

sterol	GC, RRT	relative abundance								
		type A			type B		type C			
		1	2	3	4	5	6	7	8	9
	0.91	1.9	4.0	1.0	0.7	0.7	1.7	3.4	0.5	1.0
	0.95	2.3	1.5	2.0	0.9	1.0	0.7	2.8	1.6	1.8
	1.00	28.6	24.6	19.9	17.9	6.0	14.4	17.2	19.9	19.4
	1.02		10.9		6.2	4.0	3.3	4.2	4.1	2.3
	1.11	11.5	11.2	12.9	6.5	5.9	9.2	13.8	10.1	10.3
	1.20	9.8	7.7	8.4	1.1	0.3				
	1.29	5.5	5.2	6.0	3.7	3.3	1.1	1.2	1.3	1.9
	1.36	2.2	2.5	2.0	1.3	1.7	5.1	4.5	5.9	4.8
	1.44	11.9	12.3	13.4	6.9	10.5	1.0	0.5	0.8	0.6
	1.47	25.5	18.7	31.5	3.1	3.4	10.7	10.0	9.5	12.1
	1.55						32.5	23.9	28.9	26.7
	1.76				45.5	54.4				
	1.84						9.1	5.8	9.6	9.7
	1.94						3.6	2.6	2.5	2.6

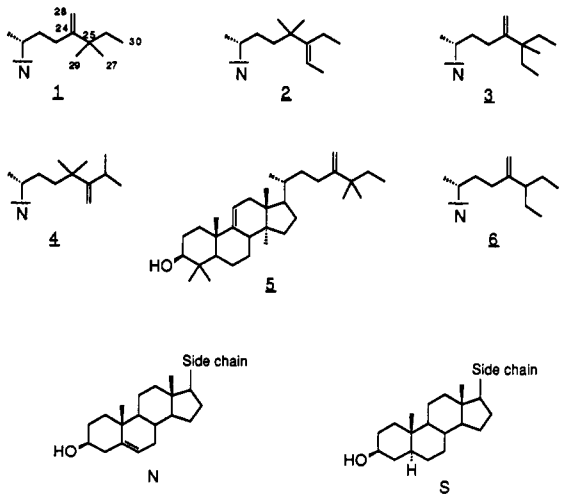


Figure 1.

sterol (6) from "the Caribbean sponge *Xestospongia muta*." It is currently believed that the two forms of *X. testudinaria* in Australia are two separate species,[16] although a more thorough taxonomic investigation is required. In view of the very similar morphology of *X. testudinaria* and *X. muta*, as well as the similarity in the sterol compositions of the two forms of *X. testudinaria* with *X. muta* types A and B, it appears that the three "types" of *X. muta* may actually be three separate species, with two of these already found in Australia.

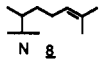
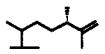
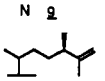
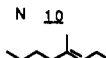
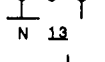
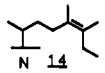
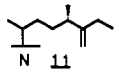
A series of incorporations with ^{3}H-labeled sterols were performed on fragments (ca. 15 cm^3) from one of the mutasterol-containing sponges (sponge no. 6 in Table I). The sponge was cut into fragments in situ, attached to labeled plastic plaques, and allowed to heal prior to being used for the biosynthetic experiments. Each labeled precursor was administered to a separate sponge fragment, and all experiments were performed in duplicate. The purity of labeled precursors was checked by HPLC prior to being used for feeding experiments. Sterol precursors were either transformed to one or more of the sponge sterols or recovered unchanged, thus indicating that all labeled substrates were incorporated in the sponge tissue. Individual sterols of the sponge were purified to constant activity by repeated injection on reversed-phase HPLC with two different solvent systems (see Experimental Section).

Due to early difficulties in keeping the sponge fragments alive during the incubation period, four separate methods for introducing each precursor to the sponge were used. Three of the four methods provided satisfactory results; these different methods are discussed in the Experimental Section.

The incorporation experiments clearly demonstrate the biosynthetic pathways operating in *X. muta* (see Scheme

(16) P. Bergquist (University of Auckland), personal communication.

Table II. Incorporation Experiments in *Xestospongia muta*

precursor[a]	radioact. fed, dpm	recovered radioact. in precursor, dpm	recovered radioactivity: mg, dpm, %[b]		
			Z	1	17
 8	4.4×10^7	3.6×10^5	62, 1.6×10^5, 25	7, 3.3×10^4, 5	16, 4.8×10^4, 7.4
 9	4.4×10^7	1.3×10^6	42, 3.4×10^5, 21	5, 4.8×10^3, 0.3	12, 9.2×10^3, 0.6
 10	4.4×10^7	2.1×10^6	72, 4.4×10^4, 2	8, 2.2×10^3, 0.1	20, 1.6×10^3, 0.1
 13	4.4×10^7	6.4×10^5	59, cold, -	6.5, 8.3×10^3, 1.3	16, cold, -
 14	4.4×10^7	1.1×10^6	24, cold, -	3, 9.7×10^3, 0.9	7, cold, -
 11	4.4×10^7	4.8×10^5	46, cold, -	5, cold, -	13, 1.1×10^5, 2.3
 12	4.4×10^7	9.0×10^5	55, cold, -	6, cold, -	15, 8.4×10^4, 9.3
squalene[d]	8.8×10^7	NA[c]	65, 4.2×10^4, -[c]	7, 3.6×10^3, -	18, 4.5×10^3, -

[a] 20 μCi of each precursor was administered to the sponge fragments; for comparative purposes, all values were obtained by using method II. For administration of precursor, see Experimental Section. [b] % refers to the percentage conversion of the precursor into the sterol based on the total recovered activity in the sponge fragment. [c] Value not available. [d] See ref 24.

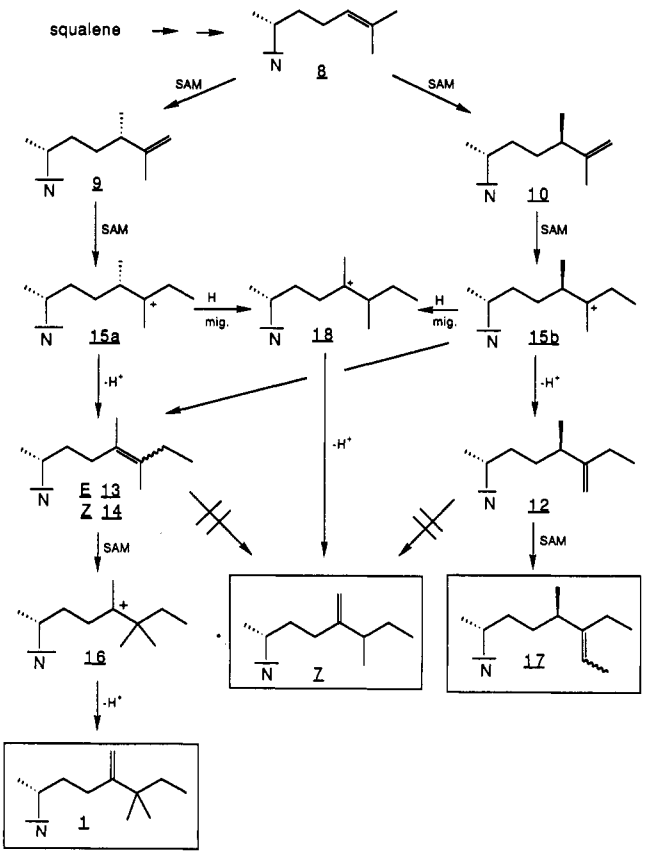
I). As we predicted in our earlier report,[10] desmosterol (8), codisterol (9), and epicodisterol (10) were transformed into the three unconventional sterols 1, 7, and 17 of this sponge (see Table II). *X. muta* exhibits considerable stereoselectivity in the second biomethylation, as codisterol (9) was ten times more efficiently metabolized to 24-(28)-dehydroaplysterol (7) than its C_{24} epimer (10).[17] This selectivity is similar to the selectivity demonstrated[18] in the biosynthesis of 24(28)-dehydroaplysterol (7) in the Australian sponge *Jaspis stellifera* but the opposite stereoselectivity was observed[3] in the California sponge *Aplysina fistularis*, where epicodisterol (10) but not codisterol (9) was converted to 25(26)-dehydroaplysterol (12). This is not surprising, since 12 retains the original C_{24} stereochemistry of the precursor, while it is lost in 7.

A lack of selectivity was observed in the SAM (*S*-adenosylmethionine) biomethylation of the *E* and *Z* isomers of 24,26-dimethyldesmosterol (13 and 14), as both precursors were equally well transformed to mutasterol (1). It is now evident that mutasterol is produced by the SAM biomethylation of desmosterol (8) to codisterol (9) (and apparently to a lesser extent epicodisterol (10)), followed by a second biomethylation to cation 15. Loss of a proton gives 24,26-dimethyldesmosterol (13/14), which then undergoes a third SAM biomethylation at C_{25} to give the C_{24} cation 16, followed by loss of a C_{28} proton with generation of mutasterol (1).

While the 24*S* configuration seems to be the preferred one in the biosynthesis of dehydroaplysterol 7, verongulasterol (17) must be produced from a different progenitor as it has the 24*R* configuration. As shown in Table II, epicodisterol (10) and 25(26)-dehydroaplysterol (12), both with the 24*R* configuration, are transformed to verongulasterol (17). Interestingly, in the feeding experiments of codisterol (9) and 25(26)-dehydroaplysterol (11), both with the 24*S* configuration, radioactivity was found in the verongulasterol (17) fraction. Precursors 9 and 11 are presumably transformed to the C_{24} epimer of verongulasterol, which co-migrates on HPLC with sterol 17.

No conversion of either isomer (11, 12) of 25(26)-dehydroaplysterol to 24(28)-dehydroaplysterol (7) was observed. This demonstrates that, rather than a hydro-

Scheme I



(17) This ratio of transformations was reproduced in a duplicate set of experiments.

(18) Cho, J.-H.; Thompson, J. E.; Stoilov, I. L.; Djerassi, C. *J. Org. Chem.* **1988**, *53*, 3466.

genation/dehydrogenation sequence[19] as was intimated in our earlier report,[10] **7** arises from a hydrogen migration of **15** to the cation **18**, followed by loss of a proton (Scheme I).

In a recent report,[20] we demonstrated that many sponges are capable of de novo synthesis of sterols. *X. muta* is one of the sponges in which de novo sterol biosynthesis was shown to operate. In the present study, we examined the incorporation of ^{3}H-labeled squalene, the acyclic sterol precursor.[21] Significant radioactivity was found in 24-(28)-dehydroaplysterol (**7**), verongulasterol (**17**), and mutasterol (**1**), demonstrating that these three unconventional sterols of *X. muta* are all synthesized de novo. *It is noteworthy that in the squalene and desmosterol (8) incorporations, with the exception of cholesterol, no radioactivity was detected in any of the conventional sterols, thus leading to the conclusion that these sterols are derived from the diet.*

Experimental Section

General. Normal-phase columns (Altex, Ultrasil-Si, 10 mm i.d. × 25 cm) with 6% ethyl acetate in hexanes as mobile phase were used to obtain a clean sample of the Δ^5-sterols. Reversed-phase columns (Altex, Ultrasphere ODS, 10 mm i.d. × 25 cm) with methanol as mobile phase, provided an initial purification of the sterols. Some of these fractions were impure and required a second reversed-phase HPLC injection with acetonitrile/methanol/ethyl acetate (11:4:3). The purity of HPLC fractions was determined by capillary GC using an FID and an HP Ultra 2 capillary column (0.32 mm i.d. × 25 m with 0.52-μm film thickness). The temperature program was 280 °C (1 min), 1°/min to 290 °C (40 min).

Synthesis of Labeled Precursors. The synthesis of the "cold" precursors used in this study has been described elsewhere: desmosterol (**8**),[22] codisterol (**9**),[3] epicodisterol (**10**),[3] 24(25)-(*E*)-dehydroaplysterol (**13**),[23] 24(25)-(*Z*)-dehydroaplysterol (**14**),[23] (24*R*)-25(26)-dehydroaplysterol (**12**),[3] (24*S*)-25(26)-dehydroaplysterol (**11**).[3] The sterols **8**–**14** were labeled with ^{3}H at C_3 by a previously described procedure,[8b,22] whereas squalene was labeled at C_3 and C_{24}.[24]

Collection of Sponges for Biosynthetic Experiments. *X. muta* was collected at a depth of 30 m, ca. 8 km from the shore of the south west coast of Puerto Rico near the Isla Magueyes Marine Station of the University of Puerto Rico at La Parguerra. Small fragments of nine individuals were collected to locate a specimen of *X. muta* that contained mutasterol. Each of these sponges was labeled with a numbered tag for later identification; the fragments were allowed to heal prior to being used in the biosynthetic experiments.

Incorporation Techniques. Four separate methods were used to introduce each precursor to the sponge fragments.[25] All experiments were kept on an underwater grid at the collection site for 4 weeks prior to harvesting and extraction.

Method I. Sponge fragments were removed from the ocean and kept in ca. 1000 mL of seawater. The precursor in 1 mL of EtOH was transferred to the seawater and the sponge allowed to filter this water for 5 h. (Early experiments had demonstrated that *X. muta* does not survive being kept in this environment for our usual[26] 12 to 24 h.) The sponge fragments were stored in a large aquarium with circulating seawater overnight and then transferred to the underwater grid at the collection site.

Method II. This is a modification of a technique developed by Scheuer et al.[27] The precursor in 3 mL of EtOH/seawater (1:1) was injected in ca. 10 "micro-injections" to different parts of the sponge fragment in situ.

Method III. A 100-μL ether/ethanol (1:1) solution of the precursor was slowly injected via miniature osmotic pumps[28] over ca. 24 h in situ. The pump was attached to the sponge fragment's plaque by a plastic cable tie.

Method IV. A solution of the precursor was transferred to a gelatin capsule and the solvent evaporated. The gelatin capsule, which slowly dissolved in water, and a sponge fragment were sealed in a ziplock bag in situ and this was attached to the underwater grid at the collection site. After 24 h, the sponge fragments were removed from the ziplock bags and reattached to the underwater grid.

All of the sponge fragments from methods II and III were alive and healthy after 4 weeks. Six of the eight fragments from method I were healthy, and only one fragment from method IV appeared reasonably healthy after 4 weeks. The total incorporation in the healthy fragments from the first three methods were fairly similar; a range of 3×10^5 to 2×10^6 dpm was reached in each instance.

Acknowledgment. We are very much indebted to Mr. George Mitcheson for his assistance with field work and Dr. Tom Tosteson for logistical support in Puerto Rico, Annemarie Wegmann-Szente for low resolution mass spectra, and the National Institutes of Health (Grant No. GM-06840) for financial assistance. Use of the 400-MHz NMR instrument was made possible by NSF Grant CHE 81-09064.

Registry No. **1**, 75886-12-3; **6**, 71031-58-8; **7**, 38636-50-9; **8**, 313-04-2; **9**, 52936-69-3; **10**, 71486-08-3; **11**, 70354-61-9; **12**, 70284-75-2; **13**, 71473-61-5; **14**, 71473-62-6; **15a**, 129466-84-8; **15b**, 129567-10-8; **16**, 129466-85-9; **17**, 75918-25-1; 27-norergosta-5,22-dien-3-ol, 64783-84-2; cholesta-5,22-dien-3-ol, 34347-28-9; cholesterol, 57-88-5; cholestan-3-ol, 80-97-7; ergosta-5,22-dien-3-ol, 474-67-9; ergost-5-en-3-ol, 4651-51-8; stigmasta-5,22-dien-3-ol, 481-16-3; stigmast-5-en-3-ol, 83-46-5; stigmasta-5,24(*Z*)(28)-dien-3-ol, 481-14-1; stigmasta-5,24(*E*)(28)-dien-3-ol, 17605-67-3; squalene, 111-02-4.

(19) Knapp, F. F.; Goad, L. J.; Goodwin, T. W. *Phytochemistry* **1978**, *16*, 1683.
(20) Kerr, R. G.; Stoilov, I. L.; Thompson, J. E.; Djerassi, C. *Tetrahedron* **1989**, *45*, 1893.
(21) For a discussion of the cyclization of squalene, see ref 14, Chapter 7.
(22) Dayal, B.; Salen, G.; Tint, G. S.; Biswas, C. *Steroids* **1983**, *42*, 635.
(23) Tarchini, C.; Rohmer, M.; Djerassi, C. *Helv. Chim. Acta* **1979**, *62*, 1210.
(24) Silva, C. J.; Wünsche, L.; Djerassi, C. To be published.
(25) For a review, see: Garson, M. *Nat. Prod. Rep.* **1989**, *6*, 143.
(26) Carballeira, N.; Thompson, J. E.; Ayanoglu, E.; Djerassi, C. *J. Org. Chem.* **1986**, *51*, 2751.
(27) Hagodone, M. R.; Scheuer, P. J.; Holm, A. *J. Am. Chem. Soc.* **1984**, *106*, 2447.
(28) Osmotic pumps were generously provided by Alza Corp., Palo Alto, CA.