4-[4-Chloro-2-methyl-5-[2-(ethoxycarbonyl)ethyl]-phenyl]butyric Acid (53a). Mp: 50–52 °C. Anal. ($C_{16}H_{21}ClO_4$): C, H.

Ethyl 3-(2-Chloro-4-methyl-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl) (54a). Mp: 63–65 °C. Anal. ($C_{16}H_{19}ClO_3$): C, H.

3-(2,7-Dichloro-4-methylnaphthalen-1-yl)propanal (56a). mp: 103–105 °C. ^1H NMR: δ 2.66 (3 H, s), 2.82 (2 H, t), 3.49 (2 H, t), 7.34 (1 H, s), 7.52 (1 H, d), 7.95 (2 H, m), 9.95 (1 H, s). Anal. ($C_{14}H_{12}Cl_2O$): C, H.

Registry No. 2, 124243-86-3; 2·Na, 124244-18-4; 3, 124243-87-4; 3·Na, 124244-19-5; 4, 124243-88-5; 4·Na, 124244-20-8; 5, 124243-89-6; 5·Na, 124244-21-9; 6, 124243-90-9; 6·Na, 124244-22-0; 7, 124243-91-0; 7·Na, 124244-23-1; 8, 124243-92-1; 8·Na, 124244-24-2; 9, 124243-93-2; 9·Na, 124244-25-3; 10, 124243-94-3; 10·Na, 124244-26-4; 11, 124243-95-4; 11·Na, 124244-27-5; 12, 108579-26-6; 12·Na, 124244-28-6; 13, 108579-36-8; 13·Na, 124244-29-7; 14, 124243-96-5; 15, 124243-97-6; 16, 124243-98-7; 17, 81945-11-1; 18, 108578-92-3; 19, 108578-93-4; 20, 10578-94-5; 21, 108578-95-6; 22, 108578-96-7; 23, 108578-97-8; 24, 108578-98-9; 25a, 108578-99-0; 25b, 108579-00-6; 26a, 108579-02-8; 26b, 108579-01-7; 27a, 108579-04-0; 27b, 108579-03-9; 28a, 108579-05-1; 28b, 124244-13-9; 29a, 108579-06-2; 29b, 124244-14-0; 31a, 108579-07-3; 31b, 108579-11-9; 33, 124243-99-8; 34, 27650-80-2; 35, 124244-00-4; 37, 124244-01-5; 38, 124244-02-6; 39, 124244-03-7; *cis*-40, 124244-04-8; *trans*-40, 124244-17-3; 41, 124244-05-9; 42, 124244-06-0; 43, 124244-07-1; 44, 124244-08-2; 45, 124266-46-2; 46, 124244-09-3; 47, 124244-10-6; 48, 124244-11-7; 49, 124244-12-8; 50a, 615-65-6; 50b, 95-69-2; 51a, 108579-27-7; 51b, 108579-13-1; 52a, 108579-28-8; 52b, 108579-14-2; 53a, 108579-29-9; 53b, 108579-15-3; 54a, 108579-30-2; 54b, 108579-16-4; 55a, 108579-34-6; 55b, 108579-22-2; 56a, 108579-35-7; 56b, 108579-23-3; Cl_3CCO_2Et, 515-84-4; $H_2^-CCOC^-HCO_2Me$, 30568-00-4; 3-hydroxy-3-methylglutaryl-coenzyme A, 1553-55-5; *N*-ethylidenecyclohexylamine, 1193-93-7; methylsuccinic anhydride, 4100-80-5; succinic anhydride, 108-30-5; 7-chloro-2,4-dimethyl-1-(3-hydroxypropyl)naphthalene, 124244-15-1; 3-(4-chloro-2-methylphenyl)propionic acid, 879-75-4; 3-(2-chloro-4-methylphenyl)propionic acid, 124244-16-2.

Lipophilic 1,3-Xylyl-21-crown-6 Macrocyclic Polyether 2-Carboxylic Acids as Biological Mimics of the Ionophore Antibiotics

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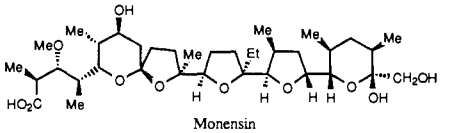
Twelve lipophilic 1,3-xylyl-21-crown-6 macrocyclic polyether 2-carboxylic acids (**9a–9l**), two lariat ether 1,3-xylyl-21-crown-6 macrocyclic polyether 2-carboxylic acids (**21** and **22**), and two 1,3-xylyl-28-crown-8 macrocyclic polyether 2-carboxylic acids (**10a** and **10b**) were synthesized and tested for in vitro antibacterial activity, in vitro stimulation of rumen propionic acid production, and in vivo anticoccidial activity in chickens. These are biological screens relevant to animal health areas where the ionophore antibiotics such as monensin have found application. While the parent structure **1** without lipophilic substituents was biologically inactive, the lipophilic macrocycles were active in the two in vitro tests but not against chicken coccidiosis. One compound (**9f**) was tested in cattle and was found to increase levels of propionic acid in the rumen fermentation. This effect is considered an important factor for increasing the efficiency of feed utilization in cattle exhibited by the ionophore antibiotic monensin. The alkali ion salts of these lipophilic macrocyclic polyether carboxylic acids are very soluble in organic solvents and insoluble in water. These compounds are proposed to act as ion-transport agents and functional mimics of the ionophore antibiotics in the biological systems described above.

The ionophore antibiotics with their fascinating array of complex structures have provided a continuing challenge to organic chemists.[1] These compounds exhibit unique activity in many biological systems via a mechanism of action which is deceptively simple: the exchange of alkali ions for protons across biological membranes.[2] Synthetic molecules which try to mimic the physical properties of the natural antibiotics have been described,[3] but only marginal success was achieved in demonstrating biological activity and no in vivo activity in either animal health area where the ionophores have made a major impact, coccidiosis control in chickens or cattle performance enhancement, has been reported. In this paper, we describe our efforts in the synthesis of polyether mimics of natural ionophores with in vivo activity in cattle and in vitro antibacterial activity.

In 1967, monensin was the first polyether antibiotic to have its structure[4] and potent biological activities,[5] such as inhibition of alkali metal cation transport in mitochondria and broad-spectrum anticoccidial activity, disclosed. It was approved for commercial use as a poultry anticoccidial in 1971 and as a cattle performance enhancer in 1975. The structure of the silver salt of monensin,[4] which is typical for the entire class, has a lipophilic exterior and a hydrophilic central cavity lined with oxygen atoms which serve as ligands for encapsulated alkali ions; the molecule as a whole is therefore neutral and lipophilic. When the carboxylate is protonated, at an interface, either biological or in solvent, the complexation of the ion, while still possible in dry, organic solvents, is weaker by several orders of magnitude[6] and the alkali ion is readily given up to the acidic aqueous layer. It is this large difference in complexation constant for alkali ions between the carboxylic acid and the carboxylate forms of the ionophore



Monensin

† Sandwich, Kent, United Kingdom.

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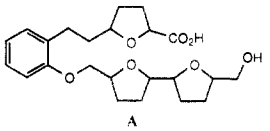
0022-2623/90/1833-0765$02.50/0

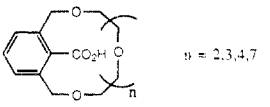
as well as rapid kinetics for ion exchange and diffusion through membranes which are the key to effective ion transport.[7]

When we started our work, we focused on the synthesis of simple acyclic analogues of monensin such as A and



A

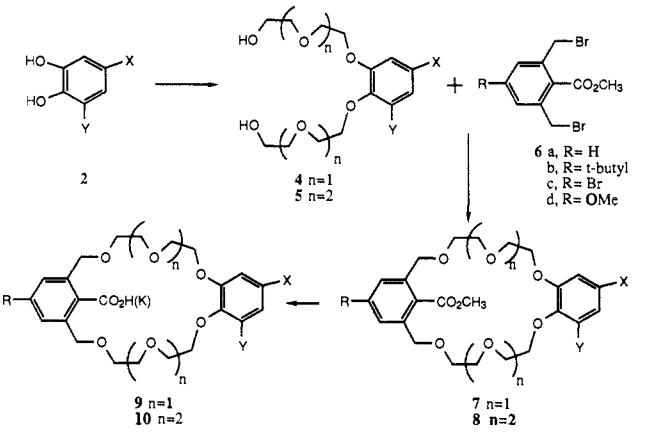
found results similar to those published by other groups,[8] some weak solubilization of alkali ions in bulk solvent layers but no significant biological activity. It appeared that the elaborate molecular architecture which preorganized the ionophore antibiotics in a cyclic conformation was a minimum requirement for effective transport of alkali ions by acyclic structures.[9] The description of similar biological activity for the enantiomer of lasalocid and the natural antibiotic was consistent with the importance of conformation over absolute stereochemistry.[10] The discovery of the crown ethers by Pederson as the first synthetic alkali ion complexing agents also occurred in 1967[11] and started a flood of elegant research on the synthesis of polyether structures as complexing agents, transport agents, and model enzymes.[12] This work included some carboxylic acid substituted compounds which could form neutral complexes with alkali ions and these served as a source of lead structures.[13] The majority of these were crown ethers to which a carboxylic acid group was appended to the periphery of the macrocyclic ring. In the cases where complexation constants were reported, both carboxylic acid as well as the carboxylate substituents served to enhance complexation relative to the unsubstituted crown ether.[13d] One structural type where this might not apply was the 1′,3′-xylyl-*m*-crown-*n*-macrocyclic polyether 2′-carboxylic acids 1 described by Cram.[14] These



1

compounds were shown to solubilize alkali ions with some selectivity, albeit weakly (<10%), and in their carboxylic acid form would be expected to be poor complexing agents since hydrogen bonding between the acid and the ring oxygens would have to be disrupted to accommodate an alkali ion in the crown ring.

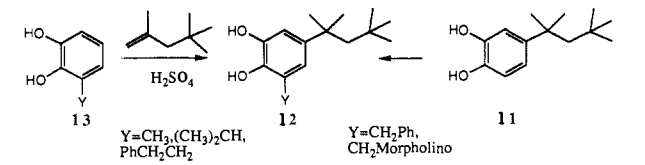
Scheme I



Chemistry

Our strategy was to introduce lipophilic groups into these macrocycles. By replacing one ethylene glycol group with catechol moiety **9a**, the synthesis was simplified and two aromatic rings were available for lipophilic substitution. For the initial compounds, symmetrical molecules were made to avoid complications from stereochemistry and the 21-membered ring was selected as suitable for complexation of sodium or potassium[13b] while being less polar than the 28-membered ring. The general macrocyclic-ring formation followed the route of Cram[14] (Scheme I). For example, catechol **2** was dialkylated with 2-[2-(2-chloroethoxy)ethoxy]tetrahydropyran (**3**) and potassium carbonate in DMF at 140 °C for 18 h. The dialkylated material was treated with 1 N HCl in methanol to remove the tetrahydropyran protecting groups and the product diol **4** was used directly in the cyclization. A solution of methyl 2,6-bis(bromomethyl)benzoate (**6a**) and diol **4** in tetrahydrofuran (THF) was slowly added to a refluxing suspension of sodium hydride in THF. Macrocyclic ester **7** was isolated by column chromatography in moderate yield, which was not optimized. Hydrolysis of methyl ester **7** was carried out in aqueous ethanol with KOH at reflux. The synthesis of 28-crown-8-macrocycles **10** utilized 2-[2-[2-(2-chloroethoxy)ethoxy]ethoxy]tetrahydropyran in the catechol alkylation step to give diols **5**.

It was possible to introduce lipophilic substituents or additional ligands onto the macrocyclic ring at various stages of the synthesis. The examples in this report were selected from a large number of compounds[15] to exemplify both the preparative possibilities and the structure–activity relationship (SAR) which we developed. The C3 and C5 positions on catechol as well as C4 of 2,6-dimethylbenzoic acid were most readily substituted. For peripheral lipophilic catechol substitution, the 1,1,3,3-tetramethylbutyl moiety (*tert*-octyl) was most useful. 4-*tert*-Octylcatechol **11** was commercially available and easily functionalized



in the C3 position. 3-Benzyl-5-*tert*-octylcatechol was

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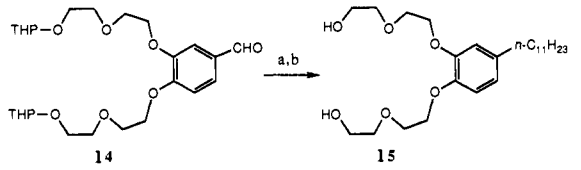
prepared in modest yield by reacting benzyl alcohol and 11 in the presence of sulfuric acid. Mannich reaction of 4-*tert*-octylcatechol with morpholine and formaldehyde in 2-propanol gave 3-(morpholinomethyl)-5-*tert*-octylcatechol.[16]

3-Substituted catechols afforded the corresponding 3-alkyl-5-*tert*-octylcatechols by sulfuric acid catalyzed alkylation with 2,4,4-trimethyl-1-pentene.[17] Both 3-methyl- and 3-isopropylcatechol were available. 3-(2-Phenethyl)-catechol was prepared from 2,3-dimethoxybenzaldehyde by treatment with triphenylphosphonium bromide and sodium hydride in DMF followed by hydrogenation (5% Pd/C, H_2, MeOH) and demethylation in 48% HBr.

In contrast with the one-step introduction of the *tert*-octyl group, *n*-alkyl groups required a multistep sequence. *n*-Decylmagnesium bromide was added to 3,4-bis[2-[2-(2-tetrahydropyranyloxy)ethoxy]ethoxy]benzaldehyde (14)

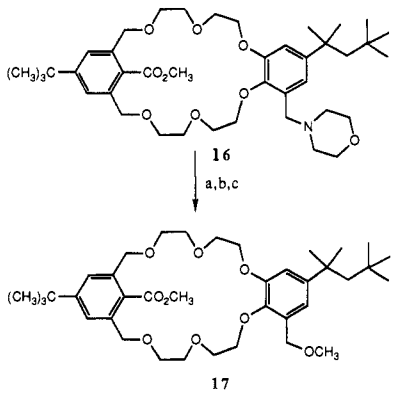


14 15

a: n-$C_{10}H_{21}$MgBr. b: H_2, 5% Pd/C, MeOH, 1N HCl

and the resulting benzylic alcohol was reduced with hydrogen in the presence of 5% Pd/C to give 1,2-bis[2-(2-hydroxyethoxy)ethoxy]-4-*n*-undecylbenzene (**15**). Using **15** in the general scheme gave **9c**.

Morpholinomethyl macrocyclic ester **16** contained a functionalized catechol. Refluxing this macrocycle in acetic anhydride caused replacement of the morpholino moiety by acetoxy.[16] Hydrolysis with 1 N KOH in ethanol at room



a: Ac_2O, reflux. b: 1N KOH, MeOH. c: NaH, Me_2SO_4, THF

temperature yielded a macrocyclic benzylic alcohol suitable for substitution. Alkylation of this alcohol with dimethyl sulfate and sodium hydride yielded the 3-methoxymethyl analogue **17**.

For the preparation of unsymmetrical macrocycles, which have been named lariat ethers by Gokel,[18] monoalkylated catechol **18** was heated neat with 1,2-epoxy-3-phenoxypropane (**19a**) or 1,2-epoxy-3-(4-*tert*-butylphenoxy)propane (**19b**) and diisopropylethylamine to give diols **20a** and **20b**, respectively. These diols were used in the standard macrocyclization and hydrolysis reactions providing **21** and **22**, respectively (Scheme II).

Scheme II

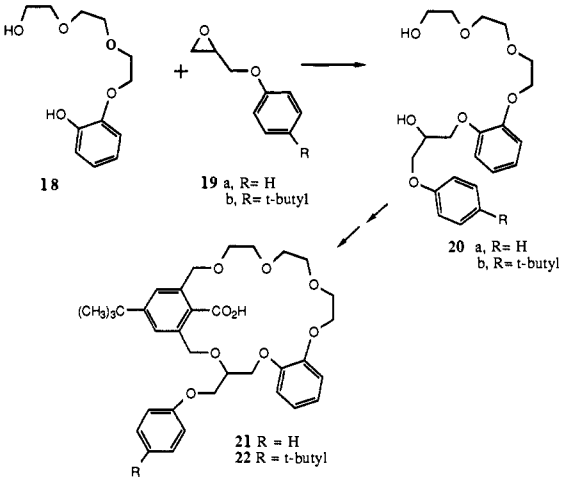


Table I

compd	substituents R	X	Y	formula[a]	% yield[b]
1 (n = 4)				$C_{19}H_{28}O_8$	17
9a	H	H	H	$C_{23}H_{28}O_8$	20
9b	H	*t*-oct	H	$C_{31}H_{44}O_8$	28
9c	H	*n*-$C_{11}H_{23}$	H	$C_{34}H_{50}O_8$	25
9d	*t*-Bu	*t*-oct	H	$C_{35}H_{52}O_8$	66
9e	H	*t*-oct	methyl	$C_{32}H_{46}O_8$	41
9f	*t*-Bu	*t*-oct	methyl	$C_{36}H_{54}O_8$	55
9g	Br	*t*-oct	methyl	$C_{32}H_{45}O_8Br$	39
9h	CH_3O	*t*-oct	methyl	$C_{33}H_{48}O_9$	32
9i	*t*-Bu	*t*-oct	CH_3OCH_2	$C_{37}H_{56}O_9$	73[c]
9j	*t*-Bu	*t*-oct	$PhCH_2$	$C_{42}H_{58}O_8$	50
9k	*t*-Bu	*t*-oct	$(CH_3)_2CH$	$C_{36}H_{58}O_8$	65
9l	*t*-Bu	*t*-oct	$PhCH_2CH_2$	$C_{43}H_{60}O_8$	37
10a	*t*-Bu	*t*-oct	H	$C_{39}H_{60}O_{10}$	41
10b	*t*-Bu	*t*-oct	CH_3	$C_{40}H_{62}O_{10}$	25
21				$C_{34}H_{42}O_9$	64
22				$C_{38}H_{50}O_9$	55

[a] Molecular formula was confirmed by high-resolution mass spectrometry on either the macrocyclic ester or free acid. All the macrocycles were >95% pure as judged by NMR and TLC. [b] Yield for the macrocyclization. [c] Yield for the alkylation of the macrocyclic benzylic alcohol.

The methyl 2,6-bis(bromomethyl)benzoates were prepared by treatment of the known methyl 2,6-dimethylbenzoates[19] with *N*-bromosuccinimide or *N,N'*-dibromo-5,5-dimethylhydantoin in refluxing carbon tetrachloride. Methyl 4-*tert*-butyl- and 4-bromo-2,6-bis(bromomethyl)-benzoates (**6b** and **6c**) were both stable solids; purified by recrystallization, while methyl 4-methoxy-2,6-bis(bromomethyl)benzoate (**6d**) was an oil which was purified by column chromatography and used immediately in the macrocycle preparation.

The parent 21-crown-6 macrocyclic carboxylic acid **9a** was isolated as a white solid. The lipophilic macrocyclic acids were all oils or glasses as free acids, but they were isolated by evaporation of a methylene chloride solution as potassium salts in the form of brittle foams which were easy to handle. The potassium salts of the 28-membered ring macrocycles **10** were somewhat hygroscopic and softened over time while the 21-membered ring macrocyclic carboxylates **9** remained free flowing. The free acid to potassium salt conversion was done by treating a methylene chloride solution of the macrocyclic acid with either 1 N HCl or 1 N KOH and isolating the desired form from

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Table II

compd	antibacterial activity: MIC, μg/mL				in vitro RPA[a]	
	S. aureus 01A005[b]	*S. zooepidemicus* 02H001	*S. equi* 02I001	*C. perfringens* 10A002	50 μg/mL	20 μg/mL
1 (n = 4)	>200	>200	>200	>200	0.0	0.0
9a	>200	>200	>200	>200	0.2	
9b	25	25	25	25	1.02	0.40
9c	1.56	1.56	1.56	3.12	0.82	0.46
9d	12.5	6.25	6.25	6.25	0.97	0.64
9e	25	50	12.5		0.87	0.33
9f	6.25	6.25	3.12	3.12	1.02	0.50
9g	12.5	12.5	6.25		0.53	0.06
9h	50	25	25		0.42	0.04
9i	12.5	25	12.5	25	0.64	0.20
9j	3.12	3.12	3.12	1.56	0.76	0.44
9k	0.78	0.78	0.78	<0.39	0.70	0.43
9l	6.25	1.56	1.56	1.56	1.09	0.32
10a	50	25	12.5	25	0.52	0.40
10b	12.5	6.25	3.12	12.5	0.41	0.11
21	25	50	25	25		0.0
22	1.56	3.12	1.56	1.56	1.06	0.6

[a] The numbers indicate the response relative to that for monensin tested at 10 μg/mL. At that level, monensin increased RPA by 36–111% compared to untreated controls. A response of 0.0 indicates no change from the control; none of the macrocycles decreased RPA levels. [b] Pfizer culture designation.

the organic layer. The compounds are listed in Table I and were characterized by their NMR, mass, and infrared spectra; the latter provided a sensitive assay for the presence of any unhydrolyzed ester in the isolated salts.

Biological Results and Discussion

The macrocycles were all tested for in vitro antibacterial and rumen propionic acid stimulation activity and in vivo anticoccidial activity. The biological results for the two in vitro screens are listed in Table II. No in vivo anticoccidial activity was found verses *Eimeria tenella* when given at 250 ppm in feed beginning 1 day before inoculation until 6 days after inoculation when the birds were necropsied to determine drug effect. The minimum inhibitory concentrations (MIC's) of the macrocyclic carboxylate salts against four Gram-positive bacteria, including one anaerobe, *Clostridium perfringens*, are shown; all were inactive against Gram-negative genera. This was the same spectrum of activity displayed by the microbial ionophores.[20] It was not surprising that the parent unsubstituted macrocycle **1** was inactive in these evaluations, since it was not expected to act as ion-transport agent. It was quite water soluble with less than 10% of the potassium salt extractable into methylene chloride.[14] However, the addition of lipophilic substituents leads to active compounds. The effect of aromatic substitution both at the periphery of the molecules as well as adjacent to the ligand cavity was studied. A group para to the carboxylate increased antibacterial activity in the order *tert*-butyl, **9f** > bromide, **9g** > hydrogen, **9e** > methoxy, **9h**, when 5-*tert*-octyl-3-methylcatechol was held constant. For good antibacterial potency, at least a C_8 straight or branched chain at the peripheral site C4(5) of the catechol was required; larger groups were more potent. However, while n-$C_{11}H_{23}$ in **9c** was a good potentiating group for antibacterial activity, this did not translate into increased potency in the rumen propionic acid screen. Also, we were concerned about the overall shape of the molecules and preferred the bulky *tert*-octyl group at C4(5) over straight-chain aliphatics. There was the possibility that macrocycles with a long aliphatic side chain might act as surfactants rather than ion-transport agents. A second lipophilic group on catechol, ortho to the ring oxygen, enhanced antibacterial potency. All alkyl and alkylaryl substituents lowered the MIC's, especially the isopropyl moiety in **9k**, while methoxymethyl caused a loss of potency.

The use of ionophore antibiotics as growth promotants in cattle[21] was based on the ability of this class of compounds to shift the balance of volatile fatty acid (vfa) production in the rumen fermentation.[22] Ruminants derive much of their energy requirements from microbial degradation of cellulose in the rumen to the vfa's (acetate, propionate, butyrate, and valerate) which are absorbed. As the relative efficiencies for the production and utilization of these acids differ, it had been postulated that "shifts" of vfa ratios toward increased production of propionic acid would lead to beneficial results on ruminant growth, and in vitro screens were developed.[23] However, until the discovery that monensin and the other microbial ionophores increased the efficiency of feed utilization in cattle, this speculation had not been reduced to practice.

In the rumen propionic acid (RPA) test procedure, a sample of rumen fluid, standard substrate, and the test compound at either 50 or 20 μg/mL were incubated in an oxygen-free atmosphere. After incubation, metaphosphoric acid was added to stop the fermentation and samples were analyzed by gas–liquid chromatography. Peak heights for acetic, propionic, and butyric acids were determined for samples from untreated and treated incubation flasks. Monensin was used as the positive control at a level of 10 μg/mL. This level of monensin was chosen to provide a maximum response for the positive control since the absolute level of propionic acid stimulation varied for each batch of rumen fluid. The range of responses with monensin varied from 36% to 111% increases in propionic acid levels. Unlike the results from antibacterial testing, in the more complex rumen fermentation, the level of RPA response in vitro reached a plateau in potency with the lipophilic 21-membered macrocyclic carboxylates once a

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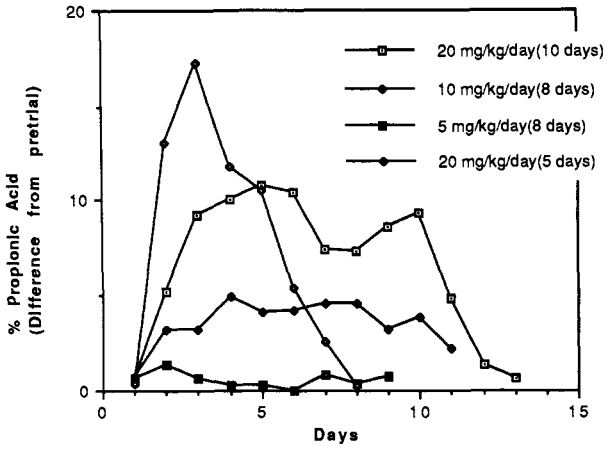


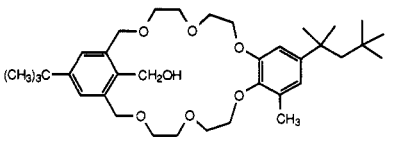
Figure 1. In vivo rumen propionic acid stimulation with **9f**.

sufficient degree of lipophilicity was achieved. While further lipophilic substitution did not increase potency; again, polar substitution, particularly methoxy **9h** and **9i**, was detrimental. The larger 28-membered macrocyclics **10a** and **10b** had parallel but weaker activity in both indications; possibly due to their greater polarity. Modifying macrocycles **10a** and **10b** to further increase lipophilicity was not explored. Lariat ether analogue **22**, stood out as an example of the beneficial effect of shielding the polar carboxylate–alkali ion complex by close-in substitution. In the case of **22**, no additional lipophilic substituent was required on the catechol moiety to achieve good activity in both in vitro screens.

Once in vitro RPA activity was found in this series of compounds, we next turned to in vivo testing. Compound **9f** was chosen for scale up as an example of one of our more active analogues as well as for its ease of synthesis since considerable material was needed. Compound **9f** was tested at 5, 10, and 20 mg/kg per day in individual animals and the results are shown in Figure 1. In this study each animal acted as its own control with base-line RPA levels measured for 1 week prior to the introduction of **9f** into the feed. As can be seen in Figure 1, both the 20 mg/kg per day dose, which was tested twice, and the 10 mg/kg per day dose increased the level of propionic acid. At 20 mg/kg per day in an 8-day trial, propionic acid levels increased as a percent of total vfa's from a mean pretrial level of 27.3% to a mean trial level of 35.1% with a peak of 38.1%. In a second 5-day trial at 20 mg/kg per day, RPA increased from 27.2% to a mean of 37.9% with a peak of 44.7%. At 10 mg/kg per day for 8 days, the increase was from 28% to 31.9% with a peak of 32.7%. At 5 mg/kg per day, no increase was found. Upon withdrawal of the drug, the propionic acid levels returned to pretrial levels. A minimum effective dose for **9f** was calculated to be 8 mg/kg per day. This can be compared to monensin which is active at <1 mg/kg per day. Although **9f** was less potent than monensin, this result marked the first time that a totally synthetic compound showed in vivo activity which mimicked that of monensin in this indication.

We have found that macrocyclic polyether carboxylic acids could act as functional mimics of the microbial ionophore antibiotics in several biological tests. While we have not proven that these crown ethers are acting exclusively by an ion-transport mechanism, several experiments were consistent with this speculation. The corresponding esters 7 and 8 were not active in the antibacterial screen. In the in vitro RPA screen, **7f** did show some weak activity at 50 μg/mL (0.3), but this could be due to a partial hydrolysis to **9f** in the complex rumen fermentation.

Benzylic alcohol **23** was inactive in all screens. These



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results point to a key role for the carboxylate group in achieving biological activity. In qualitative studies, the macrocyclic acids did transport alkali ions through bulk solvent layers,[24] but the rate of ion transport was considerably less than that for monensin. Clearly, there is much opportunity for further manipulation of structure in molecules of this size to influence partition coefficient, ion complexation and selectivity, and kinetics of ion transport. The activity found in lariat ether **23** suggests that additional improvements in potency are possible.

Experimental Section

5-*tert*-Butylxylene was purchased from Fluka Chemical Corp. 3-Isopropylcatechol, 1,2-epoxy-3-phenoxypropane, 4-*tert*-butylphenyl 2,3-epoxypropyl ether, 2-(2-chloroethoxy)ethanol, and 2-[2-(2-chloroethoxy)ethoxy]ethanol were obtained from Aldrich Chemical Co. 4-*tert*-Octylcatechol and 3-methylcatechol were purchased from K&K and Pfaltz and Bauer, respectively. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were uncorrected. NMR spectra were obtained on a Varian T-60, a Varian HA-100, or a Brucker WM 300 in deuteriochloroform ($CDCl_3$) with tetramethylsilane as internal standard. Infrared spectra were recorded on a Perkin-Elmer 283B spectrophotometer. Mass spectra were determined with a Finnigan 4510 mass spectrometer. Tetrahydrofuran (THF) was distilled from $LiAlH_4$ immediately prior to use and dimethylformamide (DMF) was used as purchased.

3-Methyl-5-*tert*-octylcatechol (2) (X = *tert*-Octyl, Y = Methyl). 2,4,4-Trimethyl-2-pentene (67 g, 0.6 mol) was added dropwise to mixture of 3-methylcatechol (56 g, 0.45 mol) and 10 drops of concentrated sulfuric acid at 100 °C over 15 min. The mixture was heated at 130 °C for 2 h and then cooled to 50 °C and ethyl acetate (500 mL) was added. The organic solution was washed with saturated sodium carbonate, 1 N HCl, and brine. An oil was recovered after drying the solution ($MgSO_4$) and evaporation. Addition of petroleum ether (150 mL) gave a white solid (76 g, 72%): mp 110–114 °C; NMR (60 MHz) δ 6.7 (s, 2), 2.2 (s, 3), 1.7 (s, 2), 1.3 (s, 6), 0.8 (s, 9); IR (CH_2Cl_2) 3530, 2950, 1475 cm^{-1}.

3-(Morpholinomethyl)-5-*tert*-octylcatechol (2) (X = *tert*-Octyl, Y = Morpholinomethyl). 4-*tert*-Octylcatechol (222 g, 1 mmol) in 2-propanol (500 mL) was added dropwise to a 60 °C solution of paraformaldehyde (40 g, 1.3 mmol) and morpholine (88 mL) in 2-propanol. The reaction mixture was stirred at reflux overnight. The alcohol was evaporated in vacuo to give a white solid which was triturated with petroleum ether and collected: 280 g; 86% yield; NMR (60 MHz) δ 8.0 (s, 2), 6.9 (d, 1), 6.5 (d, 1), 3.7 (m, 6), 2.5 (m, 4), 1.6 (s, 2), 1.3 (s, 6), 0.8 (s, 9).

Methyl 2,6-Bis(bromomethyl)-4-*tert*-butylbenzoate (6b). This was prepared according to the Cram procedure for **6a**,[14] starting from 4-*tert*-butyl-2,6-dimethylbenzoic acid.[19] The compound was crystallized from petroleum ether in 49% yield from the bromination: mp 99–100 °C; NMR (60 MHz) δ 1.35 (s, 9), 4.0 (s, 3), 4.6 (s, 4), 7.35 (s, 2).

6c: NMR (60 MHz) δ 7.7 (s, 2), 4.6 (s, 4), 4.0 (s, 3).

6d: oil; isolated by chromatography on silica gel with chloroform; NMR (60 MHz) δ 6.9 (s, 2), 4.7 (s, 4), 4.0 (s, 3), 3.9 (s, 3).

1,2-Bis[2-(2-hydroxyethoxy)ethoxy]-3-methyl-5-*tert*-octylbenzene (4f). A mixture of 3-methyl-5-*tert*-octylcatechol (23 g, 0.1 mol), 2-[2-(2-chloroethoxy)ethoxy]tetrahydropyran (45 g, 0.22 mol), and potassium carbonate (30 g, 0.22 mol) was heated in DMF (300 mL) under nitrogen at 140 °C for 20 h. The cooled reaction mixture was diluted with ether and washed four times

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with water. The ether was removed in vacuo and the resulting oil was stirred in methanol (300 mL) with 1 N HCl (5 mL) at room temperature overnight. The solvent was evaporated and the product was dissolved in CH_2Cl_2. The solution was washed with water and dried over $MgSO_4$. The product was an oil which was distilled in a Kugelrohr apparatus: 35 g; 85% yield; bp 140 °C (0.3 mm); NMR (60 MHz) δ 6.8 (s, 2), 4.3–4.0 (m, 4), 4.0–3.5 (m, 14), 2.4 (s, 3), 1.8 (s, 2), 1.4 (s, 6), 0.8 (s, 9); IR (film) 3600 cm^{-1}.

General Procedure for the Synthesis of Macrocyclic Esters. Preparation of 7f (R = *tert*-Butyl, X = Methyl, Y = *tert*-Octyl). A mixture of diol **4f** (22.6 g, 0.055 mol) and **6b** (21 g, 0.055 mol) in dry THF (200 mL) was added dropwise over 2 h to a refluxing suspension of sodium hydride (5.8 g of 50% oil dispersion, washed with petroleum ether) in THF (200 mL). The reaction was stirred overnight at room temperature and then quenched with wet THF and evaporated to an oil. The oil was dissolved in CH_2Cl_2 and washed with water and then with brine. The crude material, recovered after evaporation, was purified over silica gel by elution with 10% ethyl acetate in chloroform to give the desired product as an oil: 20 g; 55% yield; NMR (60 MHz) δ 7.4 (s, 2), 6.8 (s, 2), 4.7 (s, 4), 4.4–3.6 (m, 19), 2.3 (s, 3), 1.8 (s, 2), 1.5 (s, 15), 0.8 (s, 9); IR (film) 1720 cm^{-1}; mass spectrum, *m/e* 628.3954 (M^+); Calcd for $C_{37}H_{56}O_8$ 628.3960.

General Procedure for the Hydrolysis of the Macrocyclic Esters. Preparation of 9f (*R* = *tert*-Butyl, X = Methyl, Y = *tert*-Octyl). Macrocyclic ester **7f** (28 g, 0.045 mol) was refluxed with 1.4 M KOH in ethanol (600 mL) for 18 h, at which time TLC showed complete hydrolysis. The solvent was evaporated in vacuo and the residual oil was taken up in methylene chloride. This solution was washed with water (three times) and dried over $MgSO_4$. The filtered organic solution was evaporated in vacuo to yield the potassium salt as a brittle foam which was dried under high vacuum: 27.9 g; 95% yield (not corrected for residual water); IR (KBr) 1590 cm^{-1}. The free acid was recovered, as an oil, by washing the methylene chloride solution with 1 N HCl before drying and evaporation: IR (film) 1720 cm^{-1}; NMR (60 MHz) δ 7.4 (s, 2), 6.7 (s, 2), 4.6 (s, 4), 4.3–3.4 (m, 16), 2.2 (s, 3), 1.4 (s, 15), 0.8 (s, 9); mass spectrum *m/e* 614 (M^+, $C_{36}H_{54}O_8$).

Preparation of Macrocyclic Ester 17 (R = *tert*-Butyl, X = Methoxymethyl, Y = *tert*-Octyl). Morpholinomethyl-substituted macrocyclic ester **16** (3.82 g, 5.4 mmol) was refluxed in acetic anhydride (10 mL) for 18 h. The reaction mixture was evaporated in vacuo to remove volatiles. The residue was dissolved in ethyl ether and washed with 5% sodium bicarbonate. The crude material, after evaporation of the solvent, was chromatographed over silica gel with 10% ethyl acetate in chloroform to give the desired acetoxymethyl macrocycle: 2 g; 54% yield; IR (film) 1730 cm^{-1}; NMR (60 MHz) δ 7.4 (s, 2), 7.0 (s, 2), 5.2 (s, 2), 4.6 (s, 4), 4.4–3.5 (m, 19), 2.1 (s, 3), 1.8 (s, 2), 1.4 (s, 15), 0.8 (s, 9).

The acetoxymethyl macrocycle (2 g, 2.9 mmol) was stirred in methanol (40 mL) at room temperatre with 5% methanolic KOH (5 mL) for 2 h to yield the hydroxymethyl macrocyclic ester as an oil: 1.5 g, 81% yield; IR (film) 3400, 1725 cm^{-1}; NMR (60 MHz) δ 7.3 (s, 2), 6.8 (s, 2), 4.5 (d, 6), 4.2–3.4 (m, 19), 1.6 (s, 2), 1.3 (s, 15), 0.8 (s, 9).

The hydroxymethyl macrocyclic ester (1.5 g, 2.3 mmol) in THF (40 mL) was treated with NaH (0.17 g, 3.45 mmol, 50% oil dispersion) at 25 °C. After 5 min, a solution of dimethyl sulfate (1 mL, 10 mmol) in DMF (16 mL) was added and the reaction was refluxed for 18 h. The reaction was cooled, diluted with ether, and washed with water (three times) and brine once. The dried ($MgSO_4$) organic layer was evaporated to give **17** as an oil: 1.1 g; 73% yield; IR (film) 1725 cm^{-1}; NMR (60 MHz) δ 7.3 (s, 2), 6.9 (s, 2), 4.6 (m, 6), 4.3–3.3 (m, 22), 1.7 (s, 2), 1.4 (s, 15), 0.8 (s, 9); mass spectrum, *m/e* 658.3920 (M^+); Calcd for $C_{38}H_{58}O_9$ 658.4065.

Preparation of 1-Hydroxy-2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]benzene (18). Catechol (22 g, 0.2 mol), 2-[2-[2-(2-chloroethoxy)ethoxy]ethoxy]tetrahydropyran (50.5 g, 0.2 mol) and potassium carbonate (27.6 g, 0.2 mol) were heated in DMF (250 mL) under nitrogen at 140 °C for 18 h. The cooled reaction was diluted with ether and washed four times with water. The ether was evaporated and the residual oil was stirred in methanol (250 mL) with 1 N HCl (25 mL) for several hours. The methanol was evaporated in vacuo, methylene chloride was added, and the monoalkylated catechol was extracted into 10% aqueous KOH. The basic aqueous was acidified with 6 N HCl and crude **18** was extracted into methylene chloride. The product was purified by distillation in vacuo: 13.9 g; 29% yield; bp 170–173 °C (0.07 mm); NMR (60 MHz) δ 7.7 (s, 1), 6.9 (m, 4), 4.2–3.5 (m, 12).

Preparation of 1-[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]-2-(2-hydroxy-3-phenoxypropoxy)benzene (20a). A mixture of diol **18** (2 g, 8.4 mmol), 1,2-epoxy-3-phenoxypropane (1.27 g, 8.4 mmol), and diisopropylethylamine (0.75 mL, 8.4 mmol) was heated at 125 °C under nitrogen for 18 h. The reaction was dissolved in a 1/1 mixture of ether and methylene chloride and was washed two times with 10% KOH, two times with 1 N HCl, and once with brine. The dried ($MgSO_4$) solution was evaporated in vacuo to give the crude product, which was used directly in the macrocyclization: 2.5 g; 76% yield; NMR (60 MHz) δ 7.4–7.1 (m, 2), 6.9 (m, 7), 4.3–3.5 (m, 19).

Starting with 4-*tert*-butylphenyl 2,3-epoxypropyl ether and diol **18** gave **20b** in 73% yield: NMR (60 MHz) δ 7.3 (m, 2), 6.8 (m, 6), 4.3–3.6 (m, 19), 1.4 (s, 9).

Determination of in Vitro Antibacterial Activity. The minimum inhibitory concentration (MIC) was determined on brain heart infusion agar (BHI; Scott Laboratories, Inc., Fiskeville, RI) by the method of Ericsson and Sherris,[25] using the multiple inoculator described by Steers et al.[26] Overnight cultures, grown in BHI broth at 37 °C, were diluted 100-fold for use as the standard inoculum (20000–10000 cells in approximately 2 μL were placed on the agar surface; 20 mL of BHI agar/dish). Twelve 2-fold dilutions of the test compound were employed, with initial concentrations of the test drug being 200 μg/mL. Single colonies were disregarded when reading plates after 18 h at 37 °C. Evaluations against *Clostridium perfringens* were done by incubation under an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in an anaerobic chamber. The susceptibility (MIC) of the test organism was accepted as the lowest concentration of compound capable of producing complete inhibition of growth as judged by the naked eye. The bacterial pathogens used included one isolate each of *Staphylococcus aureus*, *C. perfringens*, *Streptococcus equi*, and *Streptococcus zooepidemicus*. *S. aureus* and *C. perfringens* were isolated from human clinical material, while *St. zooepidemicus* and *St. equi* were isolated from animal clinical sources.

Determination of in Vitro Rumen Propionic Acid Stimulation.[27] Rumen fluid was collected from a fistulated cow which was fed on a commercial fattening ration plus hay. The rumen fluid was immediately filtered through cheese cloth, and 10 mL was added to a 50 mL conical flask containing 400 mg of standard substrate (68% corn starch + 17% cellulose + 15% extracted soybean meal), 10 mL of a pH 6.8 buffer, and the test compound. The flasks were gassed with oxygen-free nitrogen for about 2 min and incubated in a shaking water bath at 39 °C for about 6 h. All tests were conducted in triplicate. After incubation, 5 mL of the sample was mixed with 1 mL of 25% metaphosphoric acid. After 10 min, 0.25 mL of formic acid was added and the mixture was centrifuged at 1500 rpm for 10 min. Samples were then analyzed by gas–liquid chromatography.[28] Peak heights for acetic, propionic, and butyric acids were determined for samples from untreated and treated incubation flasks.

Determination of in Vivo Rumen Propionic Acid Stimulation. Samples of rumen fluid from ruminating cattle (365–400 kg weight) were assayed daily as described for the in vitro RPA test for 1 week to establish base-line levels for total volatile fatty acids and for propionic acid. The test drug **9f** was administered in feed and vfa levels were determined daily with sampling done by either fistula or stomach tube. Sampling was continued for several days after removal of **9f** from the feed and in each case the percent RPA returned to the pretrial base-line level.

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for carrying out the preparation of the compounds presented in this paper. We thank also Richard S. Ware for providing high-resolution mass spectra and Dr. Earl B. Whipple for NMR studies.

Registry No. 1 (n = 4), 55440-84-1; **2** (X = t-oct, Y = CH_3), 2563-08-8; **2** (X = Y = H), 120-80-9; **3**, 54533-84-5; **4f**, 119319-62-9; **6b**, 119319-00-5; **6c**, 124175-43-5; **6d**, 124175-44-6; **7f**, 119318-69-3; **7f** (Y = CH_2OCOCH_3), 119271-39-5; **7f** (Y = CH_2OH), 119271-40-8; **9a**, 124175-32-2; **9b**, 119362-70-8; **9c**, 119362-73-1; **9d**, 119362-75-3; **9e**, 124175-33-3; **9f**, 119362-79-7; **9f**·K, 124175-45-7; **9g**, 124175-34-4; **9h**, 124175-35-5; **9i**, 124175-36-6; **9j**, 124175-37-7; **9k**, 124175-38-8; **9l**, 124175-39-9; **10a**, 124175-40-2; **10b**, 124175-41-3; 11, 1139-46-4; **12** (Y = CH_2 morpholino), 119319-02-7; **13** (Y = CH_3), 488-17-5; **16**, 119271-46-4; **17**, 119271-41-9; **18**, 65659-36-1; **19a**, 122-60-1; **19b**, 3101-60-8; **20a**, 119318-91-1; **20b**, 119319-37-8; **21**, 124175-42-4; **22**, 124199-83-3; CH_2=$C(CH_3)C$-$H_2C(CH_3)_3$, 107-39-1; 4-*tert*-butyl-2,6-dimethylbenzoic acid, 58537-98-7; morpholine, 110-91-8; 2-[2-[2-(2-chloroethoxy)ethoxy]ethoxy]tetrahydropyran, 85539-28-2.

Voronoi Binding Site Model of a Polycyclic Aromatic Hydrocarbon Binding Protein

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A three-dimensional Voronoi binding site model has been formulated from a series of competitors for the binding site on a recently isolated polycyclic aromatic hydrocarbon binding protein (PBP) from mouse liver. The PBP binds polycyclic aromatic hydrocarbons, such as benzo[a]pyrene (B[a]P), with high affinity and shows other characteristics associated with receptor–ligand complexes. Altogether, the in vitro binding constant of seven molecules were used to deduce the geometry and the energetics of a possible site model consisting of five regions: one tetrahedron-shaped finite central hydrophobic pocket, one infinite region representing access to the solvent, and three strongly repulsive regions representing the sterically forbidden walls of the pocket. The model then predicted the binding energies correctly for nine additional competitors and suggests that competition of monoaromatic (benzene) derivatives with B[a]P would be weak.

In order to understand the specific binding of small molecules to biological receptors, we have recently devised a novel approach to objectively deduce the structure and energetics of a binding site, given the observed binding energies for a series of ligands.[1-4] For example, what can we say about the shape and intermolecular forces governing the binding of competitive inhibitors of an enzyme, given their binding constants, but without knowing the enzyme's X-ray crystal structure? The algorithm for deducing this should be as little influenced as possible by the preconceptions of the investigator and should yield a vague result when given insufficient data. The technique is to construct a simplified picture of the site which still allows the ligands to explore their full range of energetically allowed conformations and alternate orientations within the site. Binding energies are modeled as a sum of interactions between ligand atoms and the regions of the site they occupy. The method we have used to achieve this is based on modeling the site as Voronoi polyhedra, and its main features have already been described.[2,4]

As a challenging test of the method, we selected a data set consisting of the binding constants of a series of competitors for a recently isolated protein, polycyclic aromatic hydrocarbon binding protein (PBP),[5] from mouse liver which binds polycyclic aromatic hydrocarbons (PAHs) with high affinity.[6-8] This protein is relatively small (31 000 Da) and binds PAHs with receptor-like properties, that is, not only with high affinity, but also in a saturable and specific manner. Benzo[a]pyrene (B[a]P) is the best characterized ligand, and other ligands have been studied as competitors for [^{3}H]B[a]P. Results with a specific binding photoaffinity label suggest that PBP has one binding site for this class of ligands.[7] The on- and off-rate binding kinetics are also consistent with a receptor function for this protein. As can be seen in Chart I, the ligands in this study are not members of any homologous series and differ widely in shape. Most methods for developing quantitative structure–activity relationships (QSAR) deal with sets of compounds which are structurally so closely related that one generally assumes they may be unam-

Table I. Observed and Calculated Binding of the Compounds of Chart I for the Five-Region Site Shown in Figure 1

compound	ΔG^a_{m-}	ΔG^a_{m+}	$\Delta G^a_{m,calc}$	optimal mode[b]
1, benzo[a]pyrene	17.7	19.0	19.0	15 C, 8 H
2, dibenzo[a,c]anthracene	17.3	18.6	18.6	14 C, 8 H
3, chrysene	16.7	18.0	17.9	14 C, 8 H
4, pyrene	16.3	17.6	16.3	13 C, 7 H
5, cyclopenta[c,d]pyrene	15.6	17.6	15.8	13 C, 6 H
6, fluoranthrene	16.7	18.0	17.8	14 C, 8 H
7, 3-methylcholanthrene	16.1	17.4	17.4	13 C, 8 H
8, 1-aminonaphthalene	10.6	11.9	11.9	10 C, 6 H (NH_2 in r_1)
			11.9	9 C, 6 H (NH_2 in r_2)
9, 2-aminofluorene	13.8	15.1	14.4	11 C, 7 H (NH_2 in r_2)
10, 1-aminoanthracene	14.9	16.2	15.2	12 C, 7 H (NH_2 in r_2)
11, 9-aminophenanthrene	14.9	16.2	15.2	13 C, 7 H (NH_2 in r_1)
			15.2	12 C, 7 H (NH_2 in r_2)
12, 9-hydroxybenzo[a]-pyrene	18.2	19.5	18.3	15 C, 7 H (OH in r_1)
13, 7α,8β-dihydroxy-7,8-dihydrobenzo[a]pyrene	17.3	18.6	18.4	12 C, 7 H (OHs in r_2)
14, 9β,10α-dihydroxy-9,10-dihydrobenzo[a]pyrene	17.6	18.9	18.9	13 C, 7 H (OHs in r_2)
15, 4α,5β-dihydroxy-4,5-dihydrobenzo[a]pyrene	17.4	18.7	17.4	10 C, 7 H (OHs in r_2)
16, 2α,3β-dihydroxy-2,3-dihydrofluoranthene	16.5	17.8	17.7	12 C, 7 H (OHs in r_2)

[a] The ΔG are given as $-\ln K_i$ where K_i is the association constant of the competitor with the receptor. [b] The optimal modes are given as the number of C and H atoms lying in r_1, the rest being in r_2.

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