J|A|C|S
COMMUNICATIONS

Published on Web 07/13/2002

Synthetic Hydraphile Channels of Appropriate Length Kill *Escherichia coli*

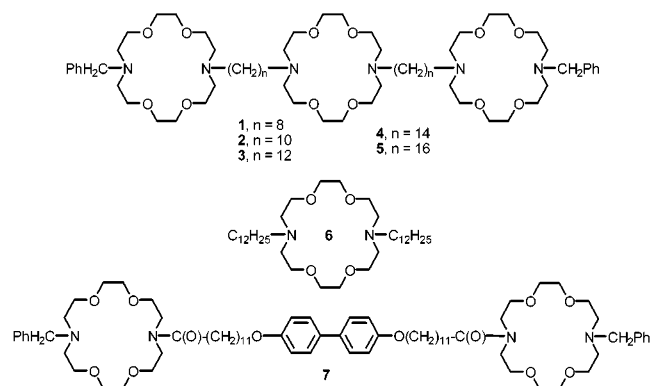
W. Matthew Leevy,[†] Gina M. Donato,[§] Riccardo Ferdani,[†] William E. Goldman,[§] Paul H. Schlesinger,[‡] and George W. Gokel*,[†]

Bioorganic Chemistry Program, Department of Molecular Biology and Pharmacology, and Department of Cell Biology, Department of Molecular Microbiology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8103, St. Louis, Missouri 63110

Received September 11, 2001

The synthetic cation conducting channels[1] that we have called hydraphiles show clear ionophoretic activity in phospholipid vesicles. Proton[2] and sodium cation transport have been assayed[3] by planar bilayer conductance methods and by use of a dynamic ^{23}Na NMR method.[4] Numerous control experiments[5] and extensive fluorescence studies[6] have established the channel, rather than the carrier, mechanism as the means by which these compounds function. One of the earliest of the channel structures in this family is shown as **3**.

The channel structure was designed[7] to possess distal macrocycles that function as headgroups in the amphiphilic sense and as entry portals for cations. The central macrocycle was intended to serve as a polar, central "relay" that would provide transient stabilization to cations. X-ray crystallographic studies have since revealed a similar structure in protein cation channels.[8] Compound **3** transports Na^+ through phospholipid bilayers at a rate about 27% that of gramicidin. When the central (macrocycle) relay is replaced by a non-hydrogen-bonding biphenyl unit (**3** to **7**), Na^+ transport is not detected by the NMR method. Likewise, the known carrier **6**, *N,N′*-bis(dodecyl)-4,13-diaza-18-crown-6, fails to transport Na^+ at a rate detectable by ^{23}Na NMR.



A number of naturally occurring peptides such as alamethicin,[9] magainin,[10] and gramicidin[11] conduct cations[12] and are known to exhibit antibiotic activity.[13] Very recently, Fernandez-Lopez and co-workers[14] showed that stacked, nanotube-forming, synthetic D,L-peptides[15] exhibit significant biological activity both against *Escherichia coli* and in red blood cells, presumably through a channel-forming mechanism. Here we report that hydraphile channel-forming compounds are active against the bacterium *E. coli* when the chain length is sufficient to span the bilayer membrane.

* To whom correspondence should be addressed. E-mail: ggokel@pcg.wustl.edu.
† Department of Molecular Biology and Pharmacology.
‡ Department of Cell Biology.
§ Department of Molecular Microbiology.

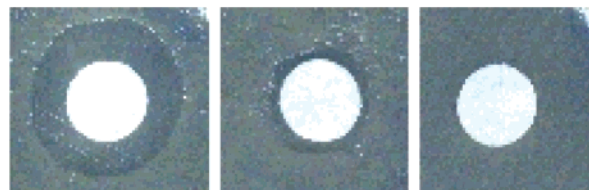


Figure 1. Disks containing **3**, **1**, and DMSO respectively on a lawn of *E. coli*.

To test the sensitivity of the Gram-negative bacterium *E. coli* to hydraphile compounds, we performed a disk diffusion assay. Bacteria were seeded onto the surface of an agar plate to form a confluent lawn. Uniformly sized cotton cellulose disks were saturated with 15 mM solutions of **1**, **3**, **6**, or **7** in DMSO, and placed on top of the bacterial lawn. After overnight growth, zones of clearing, or "halos," around the disks indicated the inhibition of bacterial growth. As shown in Figure 1, *E. coli* were highly sensitive to **3**, while compound **1** was significantly less effective. Compounds **6** and **7** exhibited no zone of clearing and gave results no different from the control disk impregnated only with DMSO. These results were confirmed using the same experimental conditions, but with a strain of *E. coli* expressing red fluorescence protein (RFP). Using 400× magnification with a Rhodamine cutoff filter (540 nm), bright-red bacterial colonies were observed growing directly underneath disks containing **6** or **7**, but not underneath those impregnated with **1** or **3**. Diazacrown **6** comprises the hydraphile backbone but functions in a membrane as a carrier rather than as a channel. Clearly, **6** is more lipophilic, should be less charged if protonated, and should insert more readily in the bilayer membrane than **3**.[16] Compound **7** lacks the central relay required for channel activity, but is otherwise very similar to **1** and **3**. These results suggest that channel function rather than hydrophobicity or other features is critical to the bactericidal activity of **1** and **3**.

Studies in synthetic phospholipid bilayers indicate that the hydraphile's ability to form a channel requires that it be long enough to span the membrane's midpolar region.[17] Synthetic hydraphiles structurally similar to **3** were systematically varied in length. The results anticipated for the experimental design were as follows. Addition or elimination of a CH_2 group alters the length of an extended chain by ±1.25 Å. An increase in the length of each dodecyl spacer in **1** by $(CH_2)_2$, that is from 12 to 14 methylenes, would increase the hydraphile's overall span by 4−5 Å. Likewise, removal of two methylene groups from each chain would diminish the span by a corresponding amount. Because these spacer chains are flexible, a 4−5 Å increase in length was expected to have only a modest effect on transport efficacy. Indeed, the transport of Na^+, by **4** as judged by ^{23}Na NMR analysis, was within experimental error of the value determined for **3**. A further increase of two

10.1021/ja017052o CCC: $22.00 © 2002 American Chemical Society

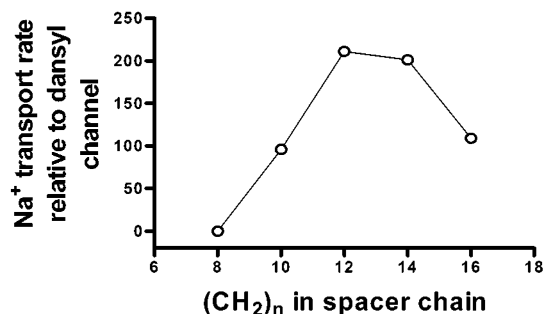


Figure 2. Na^+ cation transport vs spacer chain length for hydraphiles in synthetic vesicle systems.

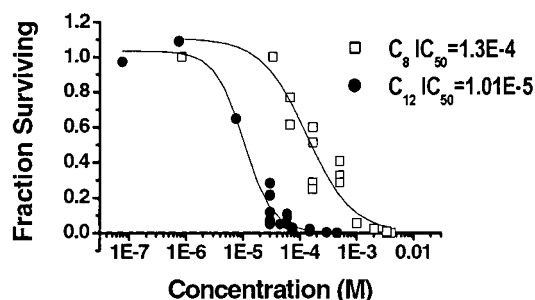


Figure 3. *E. coli* survival curves.

methylenes in each chain gave **5**, which has an overall extension approximately 10 Å greater than that of **3**. The Na^+ transport rate for **5** was found to be only 50% of that for **3**.

Decreasing the chain length had the potential for more dramatic alteration in efficacy. Since hydraphile **3** (C_{12} spacers) is known to span the bilayer,[17] incrementally shortening the structure should eventually lead to an *inactive* compound. As shown in Figure 2, the effects of shortening chain length were far more dramatic than was lengthening.

A quantitative assessment of biological activity was undertaken in bacteria by using ampicillin-resistant *E. coli.* Liquid bacterial cultures were grown to an optical density reading of 0.5 at 600 nm, indicating exponential growth. The cultures were split into 1-mL aliquots and tested against compounds having either C_{12} (**3**) or C_8 (**1**) spacer lengths, at varied concentrations. After 1-h incubations at 37 °C, aliquots were removed, serially diluted, and spread onto agar plates. The bacteria were allowed to grow overnight, after which the colonies on the plates were counted. Each colony represented one viable bacterium in the treated culture. Thus we were able to deduce the number of colony forming units in each culture and IC_{50} curves for the active compounds and controls. As shown in Figure 3, the C_{12} compound is approximately 13 times more active in killing than its C_8 counterpart. These data correspond to the at least 200-fold increase in channel activity of C_{12} over C_8 shown in Figure 2.

Fluorescence microscopy was used to visualize the location of the hydraphile in the bacterium. Dansyl channel is identical to **3** except that the terminal groups are the highly fluorescent dimethylaminonaphthalenesulfonyl (dansyl) residue rather than benzyl. Experiments were conducted as described previously for liquid culture. The experimentally optimized concentration for viewing channel insertion was approximately 12 μM, the same range as the calculated IC_{50} for **3**. For small rods the size of *E. coli* (~300 × 1500 nm), it was possible to distinguish between intracellular and surface membrane localization of dansyl hydraphiles. In general, it appeared that the fluorescent hydraphile was present primarily at the organism's periphery as shown in Figure 4. This is consistent

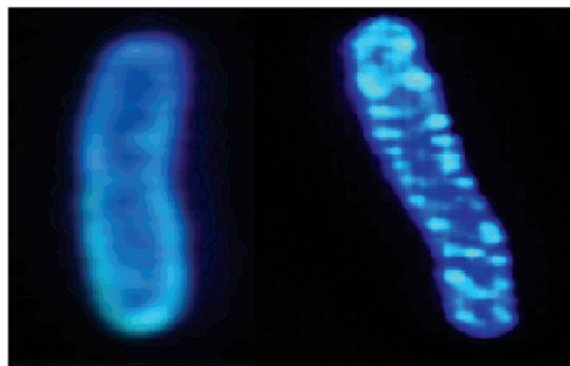


Figure 4. Examples of *E. coli* treated with dansyl channel at 12 μM. The left frame shows a cell within 30 s of treatment, the right frame shows a typical cell after 5 min.

with the channel initially incorporating into the surface membrane. With time, the peripheral membrane was populated with local regions of increased fluorescent intensity. The time-dependent change in fluorescent distribution corresponds to previous observations of an induction period for hydraphile membrane insertion and channel activation. The nature of this induction stage and heterogeneous distribution is currently under investigation.

We propose that hydraphiles exhibit toxicity to bacteria through a channel mechanism. The bactericidal activity is dependent on the presence of a functional central relay and also proper channel length. This corresponds with the structure- and length-dependent channel activity results of hydraphiles in ^{23}Na-transport studies. We speculate that hydraphiles insert into the bilayer and disrupt the cell's osmotic balance, leading to cell death.

Acknowledgment. We warmly thank the National Institutes of Health (GM 36262, T32-AI07172-22) and the Kilo Foundation for grants that supported this work.

References

(1) Gokel, G. W.; Mukhopadhyay, A. *Chem. Soc. Rev.* **2001**, *30*, 274−276.
(2) Murillo, O.; Watanabe, S.; Nakano, A.; Gokel, G. W. *J. Am. Chem. Soc.* **1995**, *117*, 7665−7679.
(3) Abel, E.; Maguire, G. E. M.; Meadows, E. S.; Murillo, O.; Gokel, G. W. *J. Am. Chem. Soc.* **1997**, *119*, 9061−9062.
(4) Riddell, F. G.; Hayer, M. K. *Biochim. Biophys. Acta* **1985**, *817*, 313−317.
(5) Abel, E.; Maguire, G. E. M.; Murillo O.; Suzuki I.; Gokel, G. W. *J. Am. Chem. Soc.* **1999**, *121*, 9043−9052.
(6) Gokel, G. W.; Ferdani, R.; Liu, J.; Pajewski, R.; Shabany, H.; Uetrecht, P. *Chem. Eur. J.* **2001**, *7*, 33−38.
(7) Gokel, G. W. *Chem. Commun.* **2000**, 1−9.
(8) Doyle, D. A.; Cabral, J. M.; Pfuetzner, R. A.; Kuo, A.; Bulbis, J. M.; Cohen, S. L.; Chait, B. T.; MacKinnon, R. *Science* **1998**, *280*, 69−77.
(9) Beven, L.; Helluin, O.; Molle, G.; Duclohier, H.; Wroblewski, H. *Biochim. Biophys. Acta.* **1999**, *1421*, 53−63.
(10) Levison, M. E.; Pitsakis, P. G.; May, P. L.; Johnson, C. C. *J. Antimicrob. Chemother.* **1993**, *32*, 577−585.
(11) (a) Burkhart, B. M.; Gassman, R. M.; Langs, D. A.; Pangborn, W. A.; Duax, W. L.; Pletnev, V. *Biopolymers* **1999**, *51*, 129−144. (b) Burkhart, B. M.; Li, N.; Langs, D. A.; Pangborn, W. A.; Duax, W. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12950−12955.
(12) Wallace, B. A. *Annu. Rev. Biophys.* **1990**, *19*, 127−157.
(13) Angelova, A.; Ionov, R.; Koch, M. H. J.; Rapp, G. *Bulg. Arch. Biochem. Biophys.* **2000**, *378*, 93−106.
(14) Fernandez-Lopez, S.; Kim, H.-S.; Choi, E. C.; Delgado, M.; Granja, J. R.; Khasanov, A.; Kraehenbuehl, K.; Long, G.; Weinberger, D. A.; Wilcoxen, K. M.; Ghadiri, M. R. *Nature* **2001**, *412*, 452−455.
(15) (a) Ghadiri, M. R.; Granja, J. R.; Buehler, L. K. *Nature* **1994**, *369*, 301−304. (b) Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. *Nature (London)* **1993**, *366*, 324−327. (c) Khazanovich, N.; Granja, J. R.; McRee, D. E.; Milligan, R.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1994**, *116*, 6677−6682.
(16) Li, C.; Budge, L. P.; Driscoll, C. D.; Willardson, B. M.; Allman, G. W.; Savage, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 931−940.
(17) (a) Murray, C. L.; Gokel, G. W. *Chem. Commun.* **1998**, 2477−2478. (b) Murray, C. L.; Gokel, G. W. *J. Supramol. Chem.* **2001**, *1*, 23−30.

JA017052O