(H_2O) and drying ($MgSO_4$), the solvent was removed and the crude product was recrystallized from EtOH/H_2O to give 1.18 g of **4**.

Biological Methods. Estradiol Receptor Binding Assay. The relative binding affinity (RBA) of the test compounds was determined by the displacement of [^{3}H]estradiol. A previously described procedure was used with modifications.[3] Test compounds were incubated with cytosol from calf uteri and [^{3}H]estradiol at 4 °C for 16 h. Incubation was stopped by adding dextran-coated charcoal. After centrifugation, the radioactivity of a 100-μL supernatant aliquot was counted. The percentage bound radioligand was plotted vs. the concentration of unlabeled test compounds. Six concentrations of the competitors were tested. They were chosen to provide a linear portion on a semilog plot, crossing the point of 50% competition. From this plot, the molar concentrations of unlabeled estradiol and of test compounds reducing radioligand binding by 50% were determined.

Estrogen and Antiestrogen Assays. Estrogenic and antiestrogenic activities were determined by stimulation of the uterine growth and the inhibition of the uterine growth stimulated by estrone, respectively, with immature NMRI mice as described previously.[3] Twenty-day-old female mice (weight 14.5 ± 1.2 g, mean ± SD) were randomly distributed into groups of 10 animals. They were subcutaneously injected daily for 3 days with 0.1 mL of olive oil solutions containing the test compound. The uteri were removed 24 h after the last injection, fixed with Bouin's solution, washed, dried, and weighed.

Mammary Tumor Growth Inhibition Test. The method used has been described previously.[3] The tumor-inhibiting effect was determined by using the DMBA-induced, hormone-dependent mammary adenocarcinoma of the SD-rat. Animals bearing at least one tumor greater than 140 mm^2 were classified in groups of 10. Compounds were dissolved in olive oil and applied sc. Measurement of tumor size and determination of body weight were made twice weekly. The therapy was continued for 28 days.

Acknowledgment. Thanks are due to the Deutsche Forschungsgemeinschaft and to the Verband der Chemischen Industrie, Fonds der Chemischen Industrie, who supported this work by grants. The technical assistance of Maria Beer and Gabi Seidl is gratefully acknowledged.

Registry No. 1, 74385-27-6; **1a**, 32445-98-0; **1b**, 7428-99-1; **2**, 96826-17-4; **2a**, 96826-32-3; **2b**, 96826-25-4; **2c**, 74457-86-6; **3**, 96826-18-5; **3a**, 96826-33-4; **3b**, 96826-26-5; **3c**, 41068-36-4; **4**, 96826-19-6; **4a**, 96826-34-5; **4b**, 96826-27-6; **4c**, 829-20-9; **5**, 96826-20-9; **5a**, 96826-35-6; **5b**, 91968-30-8; **5c**, 24826-74-2; **6**, 74385-30-1; **6a**, 74385-22-1; **6b**, 55311-42-7; **7**, 96826-21-0; **7a**, 96826-36-7; **7b**, 96826-28-7; **7c**, 96826-41-4; **8**, 96826-22-1; **8a**, 96826-37-8; **8b**, 96826-29-8; **8c**, 96826-42-5; **9**, 96844-92-7; **9a**, 96826-38-9; **9b**, 96826-30-1; **9c**, 54810-63-8; **10**, 96826-23-2; **10a**, 96826-39-0; **10b**, 72667-90-4; **10c**, 2150-40-5; **11**, 96826-24-3; **11a**, 96826-40-3; **11b**, 96826-31-2; **11c**, 73502-03-1; methyl iodide, 74-88-4.

Supplementary Material Available: ^{1}H NMR data (Table VII) of compounds **1a**–**11a** and 1–11 (3 pages). Ordering information is given on any current masthead page.

Synthetic and Conformational Studies on Anatoxin-a: A Potent Acetylcholine Agonist

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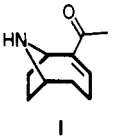
Anatoxin-a is a powerful nicotinic acetylcholine receptor agonist. Its recently reported synthesis[6] has been further optimized to provide anatoxin-a of >99% optical purity in 10% overall yield. The geometry of solid anatoxin-a has been determined by X-ray crystallography of its hydrochloride. The solution conformation has been determined by 500-MHz ^{1}H NMR spectroscopy, utilizing 2D NMR methods and homonuclear decouplings. For further comparisons, force field calculations have been employed to evaluate the differences in energy between the various conformations available for anatoxin-a. The molecule is seen to adopt the same ring conformation both in solution and in the crystal. Comparison of this conformation with the models proposed for acetylcholine receptor activation shows good agreement and allows for further inferences concerning the stereodiscrimination by the receptor.

Undisturbed transmission of the neuronal impulse over the synaptic cleft between two consecutive nerve cells is essential for normal operation of the nervous system. Impairments in acetylcholine-mediated neurotransmission can lead to severe consequences including myasthenia gravis, Parkinson's disease, and Alzheimer's disease. Therefore, the development of new efficient drugs with powerful cholinergic activity has gained increased impetus.

Good understanding of the geometrical requirements for agonist–receptor recognition is vital for rational design of new drugs with enhanced potency.[1] Inspection of various nicotinic acetylcholine agonists prompted the proposal[2] of a model for activation of the nicotinic acetylcholine receptor (nAChR).[3] This model subsequently has been refined to account for the observed stereodiscrmination at the receptor site.[4]

Anatoxin-a (1) is a low molecular weight alkaloid originally isolated from the fresh water blue-green alga *Anabaena flos-aquae* (Lyngb) de Breb.[5] The efficacy of 1 in stimulating the nAChR is greater than that of the



I

natural neurotransmitter acetylcholine. The anatoxin-a

(1) Gund, P.; Andose, J. D.; Rhodes, J. B.; Smith, G. M. *Science (Washington, D.C.)* **1980**, *No. 208*, 1425.
(2) Beers, W. H.; Reich, E. *Nature* **1970**, *228*, 917.
(3) For reviews of AChR activation, cf.: (a) Spivak, C. E.; Albuquerque, E. X. "Progress is Cholinergic Biology: Model Cholinergic Synapses"; Hanin, I., Goldberg, A. M., Eds.; Raven Press: New York, 1982; p 323 ff. (b) Maelicke, A. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 195.
(4) (a) Spivak, C. E.; Waters, J.; Witkop, B.; Albuquerque, E. X. *Mol. Pharmacol* **1983**, *23*, 337. (b) Witkop, B.; Brossi, A. "Natural Products and Drug Development", Alfred Benzon Symposium 20; Krogsgaard-Larsen, P., and Christensen, S. B., Eds.; Munksgaard: Copenhagen, 1984: p 283 ff.
(5) (a) Carmichael, W. W.; Biggs, D. F.; Gorham, P. R. *Science (Washington, D.C.)* **1975**, *No. 184*, 542. Devlin, J. P.; Edwards, O. E.; Gorham, P. R.; Hunter, N. R.; Pike, R. K.; Stavric, B. *Can. J. Chem.* **1977**, *55*, 1367.

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molecule is also sufficiently rigid in structure to allow for detailed elucidation of its conformation, both as a solid and in solution. In this report we present our findings on the conformation of 1 at physiological pH (7.2). The structure of 1·HCl also has been determined by X-ray crystallography, and its solution conformation has been examined by 500-MHz ^{1}H NMR spectroscopy. To reveal the energetics of conformational equilibria, empirical force field calculations have been performed. Also, some improvements of the recently reported[6] chirospecific synthesis of 1·HCl are described, which now allow production of 1·HCl of >99% ee in gram quantities in 14 steps and 10% overall yield from D-glutamic acid.

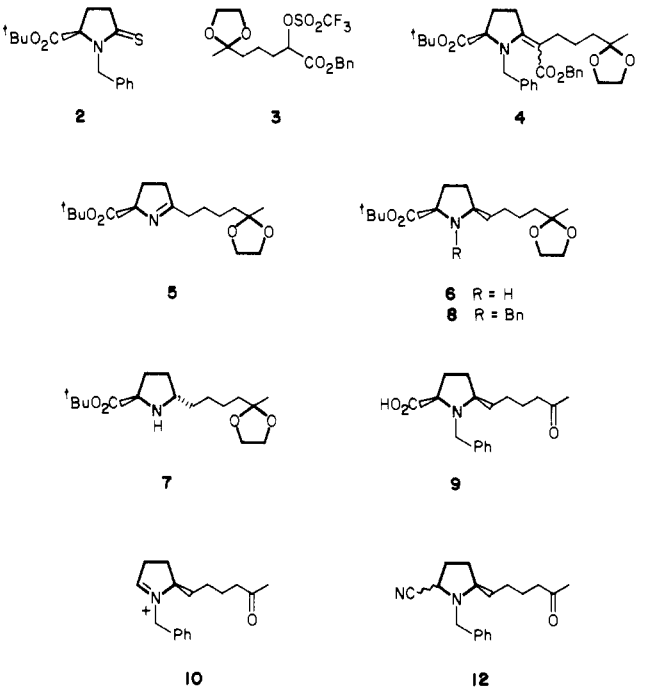
Results and Discussion

Synthesis. Anatoxin-a (1) has been the target of various syntheses leading to racemic material.[7] Recently, the total syntheses of optically active (+)-anatoxin-a (1) and its optical antipode were reported from this laboratory.[6] In the course of the present study, the need arose for further optimization of this process in regard to both the overall yield and the enantiomeric purity of the final product 1.

Optically pure crystalline thiolactam 2 is readily available from D-glutamic acid in four steps and 74% overall yield. S-Alkylation of 2 with triflate 3 followed by sulfide contraction yielded the vinylogous carbamate 4. From previous experience we knew that with only a slight excess (105 mol %) of triflate 3 the yield of 4 hovered around 60%. Some of the starting thiolactam was recovered (10–15%), and the product was usually accompanied by side products. We envisioned the problem of low conversion to reside in the stability of the triflate,[8] and indeed, use of a larger excess (120 mol %) gave a much improved yield of 4 (84% after chromatography).

The sulfide contraction reaction is also crucial since the stereointegrity of the product can potentially be diminished in this step. Proper choice of base and reaction temperature is critical. In a related study in this laboratory, *N*-methylpiperidine (pK_a 10) was found to be the base of choice and lowering the temperature to 0 °C reduced racemization tremendously.[9] In our case, at 0 °C we still encountered approximately 2% racemization but lowering the reaction temperature still further from −20 to −10 °C produced 4, which was optically pure.[10]

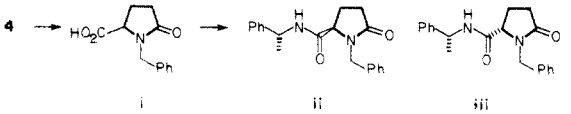
Conversion of vinylogous carbamate 4 to secondary amine 6 was then investigated. Transfer hydrogenolysis over Pd/C in methanol employing 1,4-cyclohexadiene as the hydrogen donor provided the pyrrolidine 5, which could be easily converted to pyrrolidine 6 by catalytic hydrogenation over Pt.[6] However, in a more economical process ammonium formate with much less Pd/C provided directly secondary amine 6 in yields comparable to the two-step sequence. The desired cis-disubstituted pyrrolidine 6 was invariably contaminated with approximately 2% of the trans isomer 7 as in the previous synthesis.[6] Careful chromatography was efficient in providing *cis*-6 free of *trans*-7.



Rebenzylation of 6 proceeded cleanly to provide a 93% yield of tertiary amine 8. Then, deprotection of the acid and ketone moieties in 8 was effected by acid-catalyzed hydrolysis (AcOH/*i*-PrOH/H_2O, 1/5/5), giving the solid keto acid 9.

Cyclization of 9 to the azabicyclononane 11 relies on the well-established electrophilic character of iminium ions,[11] as in 10. Since reactions of iminium ion species with nucleophiles generally can be conducted with improved yields under anhydrous conditions or, in some cases, even better with polar, nonprotic solvents and Lewis acid catalysis, we examined these alternatives. Exclusion of water by using dry methanol saturated with HCl gas was envisioned to potentiate the acid character of the reaction system, and indeed, the cyclization yield was improved from 50 to 65%. The Lewis acid approach did not prove as fruitful. Cyclization of 10 directly yielded in most cases much polymeric material. Since this might be caused by the nonvolatile byproducts formed in the preparation of iminium salt 10 by treatment of acid 9 with $POCl_3$, we adopted a slightly modified procedure. Iminium ions can be effectively trapped as the corresponding α-amino nitriles, which themselves can be easily converted back to the iminium ion species by acid (Brønsted or Lewis) treatment.[12] Thus, brief treatment of 10 with KCN in a two-phase system cleanly gave the amino nitrile 12 in 84% yield as an approximately 3/1 mixture of epimers. When 12 was sub-

(6) Petersen, J. S.; Fels, G.; Rapoport, H. *J. Am. Chem. Soc.* **1984**, *106*, 4539.
(7) (a) Campbell, H. F.; Edwards, O. E.; Kolt, R. J. *Can. J. Chem.* **1977**, *55*, 1372. (b) Campbell, H. F.; Edwards, O. E.; Elder, J. W.; Kolt, R. J. *Pol. J. Chem.* **1979**, *53*, 27. (c) Bates, H. A.; Rapoport, H. *J. Am. Chem. Soc.* **1979**, *101*, 1259. (d) Petersen, J. S.; Toteberg-Kaulen, S.; Rapoport, H. *J. Org. Chem.* **1984**, *49*, 2948.
(8) Triflate 3 eluded all attempts at purification and decomposed even on standing at 4 °C for 1 day.
(9) Shiosaki, K.; Rapoport, H. *J. Org. Chem.* **1985**, *50*, 1229.
(10) The optical purity of 4 was determined by converting it to *N*-benzylpyroglutamic acid (i) by oxidative cleavage of the



double bond followed by hydrolysis of the *tert*-butyl ester. Compound i was then converted to diastereomeric amides ii and iii with (+)-α-methylbenzylamine. Conversion from 4 to ii/iii proceeded nearly quantitatively, and HPLC analysis established the absence of iii (detection limit 0.5%).

(11) Reviewed by: Tramontini, M. *Synthesis* **1973**, 703. See also: Koskinen, A.; Lounasmaa, M. *J. Chem. Soc., Chem. Commun.* **1983**, 221 and references therein.
(12) (a) Grierson, D. S.; Harris, M.; Husson, H.-P. *J. Am. Chem. Soc.* **1980**, *102*, 1064. (b) Koskinen, A.; Lounasmaa, M. *Tetrahedron* **1983**, *39*, 1627.

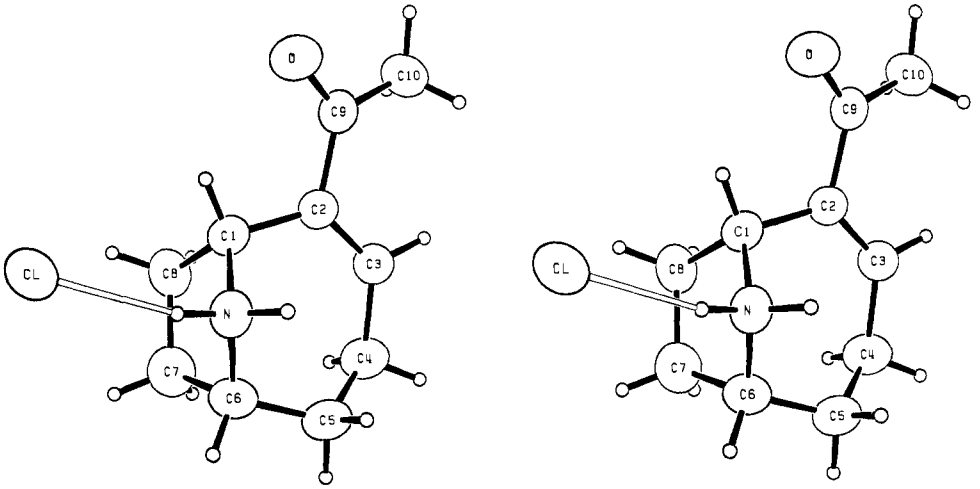
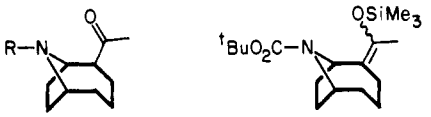


Figure 1. ORTEP stereodrawing of anatoxin-a hydrochloride (1·HCl): side view (top) and top view (bottom).

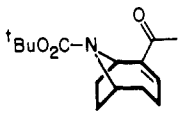
jected to the cyclization conditions (HCl/MeOH), pure **11** was formed in 80% yield.

Cleavage of the nitrogen protecting group and reprotection as the *tert*-butyl carbamate gave **14**, conversion of which to the enone **16** proved to be capricious. The Me_3Si

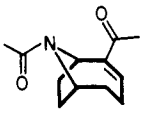


11 R = Ph
13 R = H
14 R = CO_2Bu^t

15



16



17

enol ether formation to **15** was clean, as expected. During the $Pd(OAc)_2$-mediated oxidation of **15**, however, both acetic acid and Me_3Si acetate are liberated, and substrate **15** as well as product **16** bear acid-labile groups. Indeed, closer examination of the reaction products revealed the presence of significant amounts of N-deprotected dihydroanatoxins as well as anatoxin-a itself. Triethylamine proved to be an efficient acid scavenger, and its use during oxidation improved the yield of **16** to an acceptable 52%, with 30% of **14** being recovered.

The ultimate desire to obtain a crystalline salt of anatoxin-a (**1**) was also realized. Cleavage of the BOC-protecting group from **16** and conversion of the free base **1** to its hydrochloride salt was effected as described.[6] Azeotropic removal of water from the crude hydrochloride and slow crystallization provided 1·HCl as a crystalline white solid that could be recrystallized from 3% methanol in ether to give 1·HCl of mp 152–153 °C. A sample of **1** was converted to its MTPA [α-methoxy-α-(trifluoromethyl)phenylacetic acid] amide and assayed for diastereomeric purity by HPLC as previously described.[6] The anatoxin-a (**1**) thus synthesized was determined to have an ee of >99%.

With the optimization now presented, 1·HCl can be synthesized in 14 steps with 10% overall yield, giving a crystalline product that is >99% optically pure.

Crystal Structure. Since anatoxin-a (**1**) has hitherto eluded all attempts to form crystalline salts amendable to elucidation of its conformation directly by X-ray crystallography, only the crystal structure of 2,9-diacetyl-9-azabicyclo[4.2.1]non-2-ene (*N*-acetylanatoxin-a (**17**)) is known.[13] Our expectation was that 1·HCl and **17** would differ significantly in geometry due to differences in their electronic structures, and this indeed is the case. Crystal and data collection parameters are given in Table I. Representative bond lengths and bond angles are given in Table II, along with the corresponding values reported for **17**.[3] ORTEP stereodrawings[14] of 1·HCl are presented in Figure 1, and Figure 2 gives the stereodrawing of the packing in the unit cell for 1·HCl.

Comparison of these data for 1·HCl with those reported for **17** reveals that whereas in **17** the distance across the piperidine ring at the bridge C1–N9–C6 is close to that

Table I. Crystal and Data Collection Parameters for $C_{10}H_{15}NO$·HCl, Anatoxin Hydrochloride

(A) Crystal Parameters at 25 °C[a,b]

a = 6.9846 (8) Å	space gp: $P2_12_12_1$
b = 10.7355 (12) Å	fw = 201.70
c = 14.3140 (17) Å	Z = 4
V = 1073.3 (4) Å^3	d_{calcd} = 1.248 g cm^{-3}

cryst size: 0.27 × 0.33 × 0.35 mm

(B) Data Measurement Parameters

radiation: Mo Kα (λ = 0.71073 Å)
monochromator: highly oriented graphite (2θ = 12.2°)
detector: cryst scintillation counter, with PHA
reflcns measd: +h,+k,l; 2θ range 3–45°
scan type: θ–2θ; scan speed 0.6–6.7°/min in θ
scan width: Δθ = 0.5 + 0.347 tan θ
bkgd: measd over 0.25 (Δθ) added to each end of the scan
aperture–cryst = 173 mm; vert aperture = 3.0 mm
horiz aperture = 2.0 + 1.0 tan θ mm (variable)
no. of reflcns collcd: 1629
no. of unique reflcns: 1404
intensity stds: (228), (164), (533); measd every 2 h of X-ray exposure time; over the data collcn period, no dec in intens obsd
orientation: 3 reflcns checked after every 250 measmnts; cryst orientation redetermined if any of the reflcns were offset from their predicted posns by more than 0.1°; reorientation not needed during data collcn

[a] Unit cell parameters and their esd's were derived by a least-squares fit to the setting angles of the unresolved Mo Kα components of 24 reflections with 2θ between 26 and 33°. [b] The esd's of all parameters are given in parentheses, right justified to the least significant digit(s).

(13) Huber, C. S. *Acta Crystallogr., Sect. B.: Struct. Crystallogr. Cryst. Chem.* **1972**, *B28*, 2577.
(14) Johnson, C. K. Report ORNL-3794, Oak Ridge National Laboratory, Oak Ridge, TN, 1965.

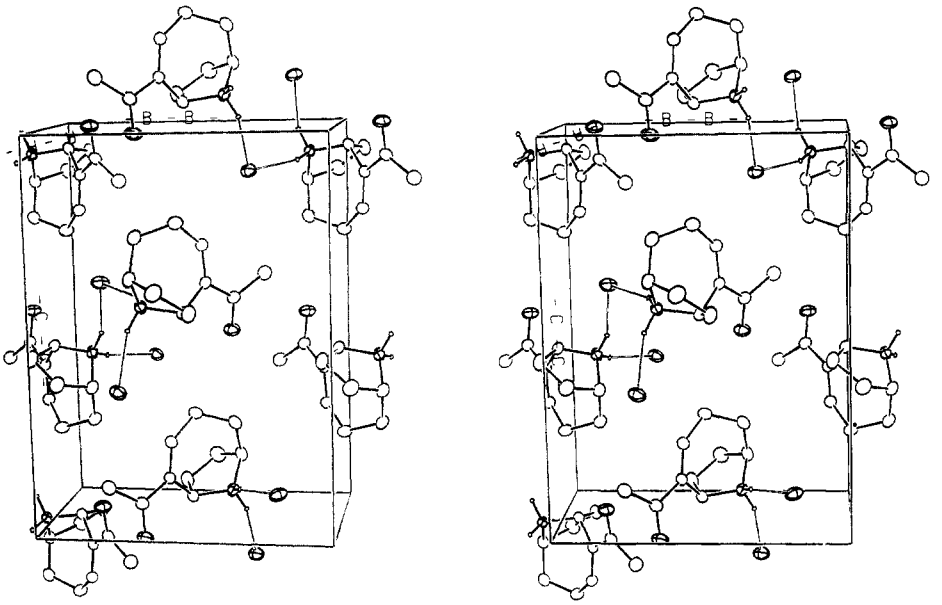


Figure 2. Stereodrawing of the packing in the unit cell of anatoxin-a hydrochloride (1·HCl).

Table II. Comparison of Selected Bond Length and Bond Angle Values for *N*-Acetylanatoxin-a (17) and Anatoxin-a Hydrochloride (1·HCl)

	17	1·HCl
	(a) Bond Lengths	
C1–C2	1.520	1.501
C1–C8	1.556	1.545
C1–N9	1.462	1.502
C2–C10	1.469	1.495
C4–C5	1.532	1.521
C6–N9	1.463	1.501
C10–C11	1.503	1.487
C10–O12	1.223	1.215
	(b) Bond Angles	
C2–C1–C8	113.6	114.3
C2–C1–N9	111.5	113.1
C8–C1–N9	102.7	101.5
C1–C2–C3	122.1	124.7
C3–C2–C10	121.9	120.1
C1–C8–C7	106.2	107.2
C1–N9–C6	109.5	106.6

reported for cocaine hydrochloride (2.39 Å vs. 2.35 Å, respectively);[13] in 1·HCl the system is even more broadened, the corresponding distance being 2.41 Å. This is seen in the increments of bond lengths C1–N9 (1.462 Å in 17, 1.502 Å in 1·HCl) and C6–N9 (1.463, 1.501 Å, respectively) and openings of the bond angles surrounding the nitrogen bridge, as shown in Table II. These effects clearly manifest the loss of amide resonance, and thus sp^2 character, of the nitrogen. As a consequence the C1–N9–C6 angle is sharpened (109.5° in 17, 106.6° in 1·HCl).

In the crystal structure of 17[13] the bond length C1–C2 (1.520 Å) is substantially longer than C3–C4 (1.496 Å). This fact was attributed to the former being between more highly substituted atoms. In the present case of 1·HCl these bond lengths are virtually identical (C1–C2 = 1.501 Å; C3–C4 = 1.497 Å), a fact that negates the above argument. As was observed for 17, and also in 1·HCl, the bond C1–C8 (1.545 Å) is significantly longer than C6–C7 (1.518 Å).

An interesting feature in the crystal structure of 1·HCl resides in the enone system (C3–C2–C10–O12). First, the bond angles surrounding C10 differ significantly from each other in 17; in 1·HCl, however, these angles are very close to equal (C2–C10–C11 = 120.5°; C2–C10–O12 = 119.9°; C11–C10–O12 = 119.6°). Furthermore, the bond length C2–C10 is significantly longer in 1·HCl than in 17 (1.495 Å vs. 1.469 Å), strongly implying reduced conjugation between the double bond C2–C3 and the ketone C10–O12. Indeed, closer inspection of the structure of 1·HCl reveals that the methyl carbon (C11) of the acetyl side chain lies 0.435 Å below the plane defined by atoms C1–C2–C3–C4 and C10. The dihedral angle between this plane and that defined by the ketone (C7–C8–C10–O12) is 17.3°, further subtantiating greatly reduced conjugation.[15]

In summary, the seven-membered ring adopts a twist-chair conformation with C4 pointing down, and the pyrrolidine ring is folded so that the nitrogen is pushed back toward the C7–C8 ethano bridge (the distance of N9 from the plane C6–C7–C8–Cl is 0.57 Å). Both the C=O and C=C double bonds are well localized as seen in the nonpolarity of the enone systems.

pK_a of Anatoxin-a (1). To gain more insight into the physical nature of 1, determination of its pK_a was undertaken. In evaluating the different methods for pK_a determinations,[16,17] we decided to conduct potentiometric titrations. The equations derived by Briggs and Stuehr[17] were employed in calculating the pK_a. Titrations were conducted on three accurately weighed samples (approximately 3 mg each) and yielded for anatoxin-a (1·HCl) a pK_a of 9.36 (±0.06). Thus, at physiological pH (7.2) 1 would be >99% protonated.

Solution Conformation. NMR Analyses. The development of high-field NMR instrumentation and pow-

(15) It is interesting also to consider the UV absorbance of anatoxin-a hydrochloride. In ethanol, typical extinction coefficients for trisubstituted enones similar to the one in 1 are usually of the order of 12000 or more. In the case of 1·HCl, the corresponding value is $\epsilon = 10700$. Assuming the simple relationship $\epsilon/\epsilon_{max} = \cos^2 \psi$ where ϵ is the observed extinction coefficient, ϵ_{max} is the exinction coefficient for the maximally overlapping planar enone system, and ψ is the deviation from planarity (the dihedral angle between the two unsaturated systems), one can calculate a value of 27° for the dihedral angle ψ, in acceptable accordance with the prediction from the crystal structure.

(16) For NMR methods, cf.: (a) Rogers, R. S.; Rapoport, H. *J. Am. Chem. Soc.* **1980**, *102*, 7355. (b) Suprenant, H. L.; Sarneski, J. E.; Key, R. R.; Byrd, J. T.; Reilley, C. N. *J. Magn. Reson.* **1980**, *40*, 231.

(17) (a) Briggs, T. N.; Stuehr, J. E. *Anal. Chem.* **1974**, *46*, 1517. (b) Meites, L.; Stuehr, J. E.; Briggs, T. N. *Anal. Chem.* **1975**, *47*, 1485.

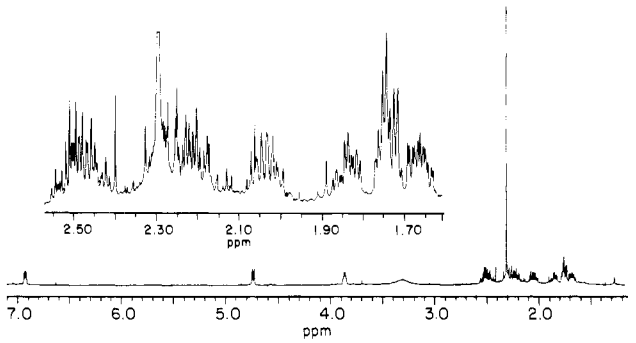


Figure 3. 500-MHz ^{1}H NMR spectrum of 1 in $CDCl_3$ at 27 °C.

Table III. ^{1}H NMR Data for Anatoxin-a (1) in $CDCl_3$ and 1·HCl in D_2O

proton	δ 1/$CDCl_3$	δ 1·HCl/D_2O	mult[b]	Δδ[a]/ppm
H-1	4.72	5.02	ddd	−0.30
H-3	6.91	7.48	ddd	−0.57
H-4α	2.45	2.63	dddd	−0.18
H-4β	2.52	2.71	dddd	−0.19
H-5α	1.72	1.92	dddd	−0.20
H-5β	1.85	2.11	dddd	−0.26
H-6	3.85	4.30	dddd	−0.45
H-7α	1.75	1.95	dddd	−0.20
H-7β	2.03	2.24	dddd	−0.21
H-8α	1.66	2.02	dddd	−0.36
H-8β	2.21	2.43	dddd	−0.22
$-CH_3$	2.29	2.37	s	−0.08

[a] $\Delta\delta = \delta(1/CDCl_3) - \delta(1\cdot HCl/D_2O)$. $\Delta\delta < 0$ signifies downfield shift. [b] Measured coupling constants (Hz): $J_{1,3} = 1.5$; $J_{1,8\alpha} = 2$; $J_{1,8\beta} = 9$; $J_{3,4\alpha} = 4$; $J_{3,4\beta} = 7.5$; $J_{4\alpha,4\beta} = 17.5$; $J_{4\alpha,5\alpha} = 4.5$; $J_{4\alpha,5\beta} = 11$; $J_{4\beta,5\alpha} = 5$; $J_{4\beta,5\beta} = 5$; $J_{5\alpha,5\beta} = 14.5$; $J_{5\alpha,6} = 2$; $J_{5\beta,6} = 3.5$; $J_{6,7\alpha} = 3.5$; $J_{6,7\beta} = 7.5$; $J_{7\alpha,7\beta} = 12.5$; $J_{7\alpha,8\alpha} = 9.5$; $J_{7\alpha,8\beta} = 4$; $J_{7\beta,8\alpha} = 6$; $J_{7\beta,8\beta} = 12.5$; $J_{8\alpha,8\beta} = 12.5$.

erful methods such as two-dimensional (2D) ^{1}H NMR experiments[18] has enormously aided the assignment of spectra of complex natural products. With the help of these techniques, conformational analysis of several biologically important natural products has become viable.[19] Although the structure of anatoxin-a (1) looks deceptively simple, its ^{1}H NMR spectrum even at the 250-MHz level exhibits complex second-order coupling patterns. Resorting to 500-MHz ^{1}H NMR spectra, we were finally able to obtain well-resolved spectra and assign all the 11-ring protons in addition to the methyl singlet and NH.

A representative 500-MHz ^{1}H NMR spectrum for 1 in $CDCl_3$ is given in Figure 3. Comparison of this spectrum with that of 1·HCl in D_2O reveals that the observed coupling patterns remain virtually identical. However, even at 500 MHz, the spectrum of 1·HCl in $CDCl_3$ exhibits, in addition to signals for H-1, H-3, H-6, the NH protons, and the methyl signal, three multiplets consisting of the remaining eight protons, thus defying all efforts to assign the spectrum completely. For such assignments, a homonuclear correlation experiment (COSY)[20] was performed on 1 in $CDCl_3$. Examination of the contour plot revealed geminal and vicinal H–H connectivities. The couplings and magnitudes of the coupling constants were further confirmed by successive homonuclear decoupling experiments. The spectral parameters for anatoxin-a (1) in $CDCl_3$ and 1·HCl in D_2O are given in Table III.

Table IV. Calculated Karplus Parameters for the Two Aliphatic Four-Spin Systems of 1

spin syst	$^3J(\theta) = A\cos^2\theta + B$ A	B	θ	r
H4α,H4β,H5α,H5β				
regression best fit (X-ray)	5.78	3.27	42.8	0.780
minimization method (solution)	8.04	2.76	60	
H7α,H7β,H8α,H8β				
regression best fit (X-ray)	6.14	4.98	2.0	0.974
minimization method (solution)	8.42	2.86	8	

Solvation effects in D_2O cause overall downfield shifts for all protons. This effect is most pronounced at the bridgehead positions, H-1 and H-6, amounting to Δδ −0.30 and −0.45 ppm, respectively. Also, the olefinic proton H-3 experiences a downfield shift of Δδ −0.57 ppm, obviously due to transmission of the solvation effect through the C2–C3 double bond. Stronger than average downfield shifts are also experienced by H-5β (Δδ −0.26 ppm) and H-8α (Δδ −0.36 ppm). The former is obviously due to proximity of the proton and the (solvated) nitrogen. The reasons for the latter are somewhat obscure, but it seems reasonable to attribute this to through-space field effects with the bridge nitrogen. The average downfield shift for the rest of the protons is −0.20 ppm, except for the methyl group, which quite expectedly is less shielded (Δδ −0.08 ppm) by solvation.

An interesting feature in the couplings is observed in the C7–C8 ethano bridge of the pyrrolidine ring. It is known that the cisoid coupling of exo and endo protons in norbornanes are not equal,[21] the exo couplings being larger ($J_{exo-exo} = 12$ Hz; $J_{endo-endo} = 10$ Hz). Recently,[22] further application of this transmission effect[23] to the analysis of 7-hetero-substituted norbornanes led to the conclusion that the ethano bridge–ethano bridge endo interaction reduces the exo–exo coupling and the ethano bridge–hetero bridge exo interaction specifically reduces the endo–endo coupling. In the case of anatoxin-a, endo interaction would be reduced to one (as compared to two in the corresponding 7-azanorbornane), and thus the exo–exo coupling should be at least 2.5 Hz larger than in the 7-azanorbornane case. Indeed, this is what was observed: $J_{7\beta,8\beta} = 12.5$ Hz and $J_{7\alpha,8\alpha} = 9.5$ Hz.

To gain information concerning the solution conformation of anatoxin-a, the Karplus relationship[24,25] of eq 1 was

$$^3J_{HH}(\theta) = 8.5\cos^2\theta - 0.28 \qquad 0° \le \theta \le 90° \qquad (1)$$

$$^3J_{HH}(\theta) = 9.5\cos^2\theta - 0.28 \qquad 90° \le \theta \le 180°$$

applied to calculate the approximate dihedral angles from the measured coupling constants. The spin systems we are most interested in, the four-spin systems C3–C4 and C7–C8, were calculated first from the dihedral angles θ derived from X-ray data and the measured coupling constants. The A and B values thus calculated are given in Table IV. Clearly, the solid-state and solution conformations are similar. However, when the appropriate vi-

(18) Recently reviewed by: Shoolery, J. N. *J. Nat. Prod.* **1984**, *47*, 226.
(19) Examples of conformational analyses of natural products relying on high-field NMR: (a) (peptide alkaloids) Lagarias, J. C.; Yokoyama, W. H.; Bordner, J.; Shih, W. C.; Klein, M. P.; Rapoport, H. *J. Am. Chem. Soc.* **1983**, *105*, 1031. (b) (indole alkaloids) Lounasmaa, M.; Kan, S.-K. *Tetrahedron* **1980**, *36*, 1607. (c) (vancomycin) Williamson, M. P.; Williams, D. H. *J. Am. Chem. Soc.* **1981**, *103*, 6580. (d) (sipholanes) Carmely, S.; Kashman, Y. *J. Org. Chem.* **1984**, *48*, 3517.
(20) (a) Bax, A.; Freeman, R.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 164. (b) Bax, A.; Freeman, R. *Ibid.* **1981**, *44*, 542.
(21) Marchand, A. P.; Marchand, N. W.; Segre, A. L. *Tetrahedron Lett.* **1969**, 5207.
(22) de Leeuw; F. A. A. M.; van Beuzekom, A. A.; Altona, C. *J. Comput. Chem.* **1983**, 438.
(23) Barfield, M. *J. Am. Chem. Soc.* **1980**, *102*, 1.

Table V. Comparison of Dihedral Angles for Anatoxin-a Hydrochloride from X-ray Data and Calculated from NMR and Force Field

dihedral angle	X-ray	NMR[a]	FF[b]
Hα-4-5-Hα	39.9	60	58
Hα-4-5-Hβ	178.6	180	173
Hβ-4-5-Hα	-93.1	-60	-56
Hβ-4-5-Hβ	45.6	60	59
Hα-7-8-Hα	-2.2	-8	-14
Hα-7-8-Hβ	86.4	112	103
Hβ-7-8-Hα	-82.3	-128	134
Hβ-7-8-Hβ	-1.8	-8	16

[a] Calculated from coupling constants with the aid of the Karplus equation.[24] [b] Obtained from force field calculations for conformers A and B.

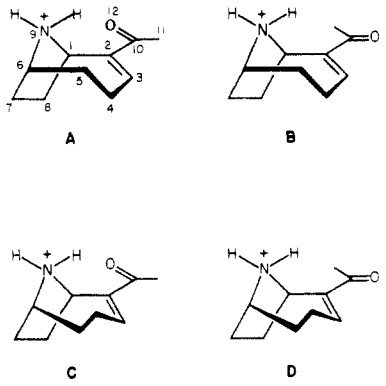


Figure 4. Possible conformations considered for 1·HCl.

cinal angles were summed, the geminal angles were found to deviate significantly (16–26°) from the value 120° expected for the projection of a tetrahedron to a plane perpendicular to the C–C axis. Therefore, we needed to develop a Karplus-type equation with the further constraint that the projection of the geminal angles be 120°. When the geminal angles are thus fixed, the vicinal angles are interrelated as follows: $\angle 4\alpha,5\alpha = \theta = \angle 4\beta,5\beta$; $\angle 4\alpha,5\beta = 120 + \theta$; $\angle 4\beta,5\alpha = 120 - \theta$. Similar equations hold for the four-spin system H7α,H7β,H8α,H8β. Minimization of the function

$$F = \sum_{i=1}^{4} (J_i^{exp} - J_i^{calc})^2 = \sum_{i=1}^{4} (J_i^{exp} - A\cos^2\theta - B)^2 \quad (2)$$

yielded the values A, B, and θ_0 given in Table IV.

The dihedral angles of the two four-spin systems C3–C4 and C7–C8 are summarized in Table V. Although any quantitative arguments on the conformation based on coupling data are to be discouraged,[24b] one can immediately see that qualitatively the crystal and solution conformations bear a striking resemblance. One can conclude that in solution, as in the crystal form, anatoxin-a attains a ring conformation that is aptly presented by structures A and B (Figure 4). To gain insight into the conformation of the enone system in solution, we performed an NOE difference experiment, irradiating the methyl singlet at δ 2.29. Very small enhancement (<5%) was observed for both H-1 and H-3, suggesting that the enone system is not locked into the s-trans conformation.

Table VI. Calculated Strain Energies (E_s), N–O van der Waals Distances,[a] and C3–C2–C10–O12 Dihedral Angles for 1·HCl Conformations A–D

conformn	E_s, kcal mol^{-1}	N–O,[a] Å	dihedral angle,[b] deg
A	24.52	4.48 (4.48)	-162.5 (-162.7)
B	23.77	6.04 (6.08)	21.0
C	25.76	5.04	-172.7
D	25.23	6.24	15.4

[a] Distance between the center of the positive charge and van der Waals surface (1.4 Å) of carbonyl oxygen as described in ref 2. The values in parentheses were obtained from X-ray data. [b] Dihedral angle C3–C2–C10–O12. When viewed along C2–C10, if O12 is counterclockwise from C3, the angle is given a negative sign.

Force Field Calculations. Computer-aided structure analysis by means of empirical force field calculations (also called molecular mechanics, MM)[26] provides information not only on the preferred conformation of the molecule but also of the energetics between various alternative conformations available for the compound. Furthermore, information about the magnitudes of different energy contributions can be obtained directly from the computer printout.

With these presumptions in mind, we also performed the force field calculations for anatoxin-a hydrochloride (1·HCl), using the MMP2 program of N. L. Allinger.[26b,c] The four possible conformations A, B, C, D, given in Figure 4 were to be considered. These conformations are noted as s-trans (s-cis) chair (boat) referring to the s-trans (s-cis) disposition of the enone system (C3–C2–C10–12) and the (twist) chair or boat conformation of the seven-membered ring. The calculated strain energies, N–O distances,[27] and the dihedral angles for C3–C2–C10–O12 are given in Table VI.

The s-cis chair conformation B was calculated to be the most stable. Rotation around the C2–C10 bond to the s-trans chair conformation A leads to a slightly less favored structure (ΔE_s = 0.75 kcal mol^{-1}). Pseudorotation of the seven-membered ring to a twist-boat conformation with C4 above the plane defined by C2, C3, and C5 leads to a higher energy conformation, again the trans form being less favored. Comparison of the values for N–O distance and the C3–C2–C10–O12 dihedral angles with those obtained from X-ray crystallographic data (Table VI, in parentheses) reveals excellent agreement.

Closer examination of the interactions reveals that the boat forms C and D are destabilized relative to the corresponding chair forms A and B mainly due to the increased compression caused by Hβ–NH, NH′–Hβ, and Hα–Hα proximities. These effects cause folding of the (envelope) pyrrolidine ring, in part releasing some of the torsional strain due to staggering around H–N–C(bridgehead)–H. However, this release is far less than the contributions imposed by compression as described above. In each ring conformation, the s-cis conformation of the enone is energetically favored. Inspection of the energy contributions again reveals this to be mainly due to release of

(24) (a) Karplus, M. *J. Chem. Phys.* **1959**, *30*, 11. (b) *J. Am. Chem. Soc.* **1963**, *85*, 2870.

(25) The more detailed analysis of K. G. R. Packler (*J. Chem. Soc., Perkin Trans. 2* **1972**, 1936) using considerably more complex calculations led to the same general results. Since, as pointed out in ref 24b, strict quantitative arguments concerning dihedral anlges based on coupling data is hazardous, we decided to rely on the computationally simpler method.

(26) (a) Osawa, E.; Musso, H. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 1. (b) Burkert, U.; Allinger, N. L. "Molecular Mechanics"; American Chemical Society: Washington, DC, 1982; ACS Monogr. No. 177. (c) The Allinger program was made available to us by Molecular Design Limited, Hayward, CA.

(27) The N–O distance refers to the structural parameter defined in ref 2 as the distance between the center of the positive charge and a point on the van der Waals surface of the hydrogen-bond acceptor. For a carbonyl oxygen as H-bond acceptor, the latter point is the point of intersection of the extension of the line C–O and the oxygen van der Waals surface (1.4 Å).

Table VII. Concentration of Some ACh Agonists That Produce a 10-mV Depolarization in Frog Sartorius Muscle

agonist	concn, μM	agonist	concn, μM
anatoxin-a	0.2–0.4	carbachol	6–10
ACh	1	nicotine	20
succinylcholine	3		

the steric strain experienced by the methyl group.

At this point it is appropriate to compare the results from each of the three methods used to examine the conformation of 1·HCl. X-ray structure, ^{1}H NMR studies, and the force field calculations all suggest the same ring conformation, with the seven-membered ring adopting a twist-chair conformation. In the solid state, the enone system adopts a uniform s-trans conformation. However, the force field calculations predict the s-cis form to be predominating, although only in about a 3/1 ratio. Indeed, as the NOE experiments revealed, the acetyl side chain is rather freely rotating. Thus, in the solid state, crystal packing forces (which are not taken into account in the calculations) force the enone into a single conformation.

Conclusions. Anatoxin-a (1) has a pK_a of 9.4 and therefore exists in the protonated form at physiological pH. The 9-azabicyclo[4.2.1]non-2-ene ring system adopts a conformation where the seven-membered ring is in a twist-chair form with C4 pointing downward (conformations A and B, Figure 4), as evidenced by X-ray crystallographic structure determination, 500-MHz ^{1}H NMR spectroscopy, and empirical force field calculations. The enone portion of the molecule exists in an s-trans conformation in the solid state. However, NMR and MM calculations lead to a different conclusion. By NMR, both s-cis and s-trans conformations are significantly populated at room temperature, and by MM calculations the ratio B/A was calculated to be approximately 3/1. It is worth noting that the conformation of the enone portion does not significantly affect the conformation of the bicyclic ring structure.

Comparison of the dihedral angle values around C4–C5 and C7–C8 derived from all three methods (Table V) shows that these values are in good agreement. Especially striking is the agreement between the values obtained from NMR and MM. The small deviations in the X-ray-derived values are attributed to crystal packing, as in the case of the enone conformation, but again the structures are in good qualitative agreement.

Anatoxin-a definitely is a powerful ACh agonist (Table VII).[28] Also, it has been estimated that the relative potency of natural (+)-AnTx-a is several orders of magnitude greater than that of its enantiomer (−)-AnTx-a. Indeed, the comparison of AnTx-a, carbachol, and succinylcholine disclosed a clearly larger potency for AnTx-a.[4] It is therefore of interest to see how well the structure of 1 compares with the models proposed for the nAChR activation.[2-4] Similar estimates have hitherto been carried out on a structure predicted by various force field calculations or based on the structure of *N*-acetylanatoxin-a. However, since no information on the solution conformation of 1 has been available until now, conclusive statements have not been possible.

As pointed out previously,[2] the distance between the electrostatic interaction (quaternized N) and the hydrogen-bonding acceptor (carbonyl O) is of paramount importance. Comparison of the values given in Table VI with the value of 5.9 Å proposed[2] reveals that the s-cis conformation B and D are in close agreement with the model. These values also compare favorable with those used to propose the active conformer to be s-cis boat (D).[4b] However, in regard to the conformation of the bicyclic ring skeleton, we are led to a different conclusion. Since the N atom will be protonated (pK_a 9.4) at physiological pH and since the solution conformations predicted by both high-field NMR and force field calculations to predominate are conformations A and B, we are led to the conclusion that the active conformation of anatoxin-a is best represented by conformation B.

The observed stereodiscrimination by the ACh receptor warrants some comments. It has already been suggested[2] that in nicotine "the role of the carbon skeleton is simply to provide proper stereochemical localization of the functional groups". Later this idea has been extrapolated to carbonyl H-bond acceptors like anatoxin-a by considering the plane defined by the hydrogen-bond acceptor.[4] Indeed, good correlation between lone-pair directionality and ligand–macromolecule binding has recently been reported.[29] Our findings on the structure of anatoxin-a are consistent with the earlier findings that the AChR donates a hydrogen bond to the agonist[2] and recognizes the plane defined by the hydrogen-bonding system.[3] The Coulombic interaction site of the agonist is positioned out of this plane at an optimal distance. For example, in ferruginine, the spatial relationships between the nitrogen atom and the enone carbonyl are significantly changed relative to anatoxin-a. Also the activity of anatoxin is almost 10^3 times that of ferruginine.[4]

Thus, with a potent acetylcholine agonist of the considerable rigidity of anatoxin-a at hand, the geometrical requirements for receptor activation can be better defined. It must, however, be borne in mind that the receptor–effector interactions are perturbational in nature. Further studies are in progress addressing the question of optimal geometry for AChR stimulation by probing the receptor with structurally more rigid agonists.

Experimental Section

Nuclear Magnetic Resonance. The 500-MHz ^{1}H NMR spectra were obtained with samples prepared by dissolving 5 mg of 1 or 1·HCl in 0.4 mL of $CDCl_3$ or D_2O, respectively. In the $CDCl_3$ spectra, 1% internal Me_4Si was used, and in the D_2O spectra, the carefully temperature calibrated HOD signal was used as reference. The temperature inside the probe was 27 °C. Peak positions were obtained from a computer printout. A pulse flip angle of 42° (5.0 μs) with 2.0-s delay between pulses and acquired spectral width of 4000 Hz (quadrature detection, Butterworth filter on) were employed. A total of 32 scans were accumulated and the FID's were signal averaged into a 32K memory block with an acquisition time of 2.05 s and 125-μs preacquisition delay time. The resulting FID was base line corrected before FT. For resolution enhancement, the FID was also apodized by double-exponential multiplication.

Prior to the acquisition of the 2D data file, the 90° pulse length was measured (17 μs). The 2D time–domain data matrix was set up by regularly incrementing t_1 of the familiar 90-t_1-90-t_2 COSY sequence[20] and accumulating the NMR response during t_2. Spectral width in both dimensions was 3800 Hz (quadrature detection). A total of 128 different values of t_1 were utilized. The data were transformed with sine-bell multiplication in both dimensions and zero filling to 1K in the F_1 dimension.

Single-Crystal X-ray Analysis of 1·HCl. Small clear colorless crystals of 1·HCl were obtained by slow crystallization from 3% methanol in diethyl ether. Preliminary precession photographs indicated orthorhombic Laue symmetry and yielded

(28) Albuquerque, E. X., personal communication.

(29) Murray-Rust, P.; Glusker, J. P. *J. Am. Chem. Soc.* 1984, *106*, 1018. For geometry of the N–H···O=C hydrogen bond, cf. also: Taylor, R.; Kennard, O.; Versichel, W. *J. Am. Chem. Soc.* 1983, *105*, 5761.

preliminary cell dimensions. Systematic absences were consistent only with the space group $P2_12_12_1$. A data set was collected on an Enraf-Nonious CAD-8 diffractometer. The raw intensity data were converted to structure factor amplitudes and their esd's by correction for scan speed, background, and Lorentz and polarization effects. The structure was resolved by the MULTAN 11/82 program. Refinement proceeded via standard least-squares and Fourier techniques. The correct enantiomer was selected on the basis of the known chirality of the molecule, and refinement against the acentric data set commenced with the location of all non-hydrogen atoms. In a difference Fourier map calculated following refinement of all non-hydrogen atoms with anisotropic thermal parameters, peaks corresponding to the expected positions of most of the hydrogen atoms were found. The hydrogen parameters were added to the structure factor calculations but were not refined. In the final cycles of full-matrix least-squares refinement an extinction coefficient[30] was refined. The final R value was 2.41%. A final difference Fourier map showed no missing or misplaced electron density.

The refined structure was plotted by using the ORTEP computer program (Figures 1 and 2).[14] Coordinates, anisotropic temperature factors, least-square planes, and structure factor calculations are available as supplementary material.

Synthesis. Tetrahydrofuran (THF) was distilled from $LiAlH_4$; acetonitrile (CH_3CN), dichloromethane (CH_2Cl_2), and N-methylpiperidine were distilled from CaH_2; methanol was dried by distilling from $Mg(OMe)_2$. Benzyl bromide and $POCl_3$ were distilled immediately prior to use. All reactions were performed under a static atmosphere (balloon) of nitrogen or argon. Organic solvent solutions were dried over Na_2SO_4 before evaporation at reduced pressure with a Berkeley rotary evaporator. Melting points were determined on an open-capillary Buchi apparatus and are uncorrected. For chromatography, silica gel 60 (E. Merck) was used. Routine ^1H NMR spectra were recorded in $CDCl_3$ solutions, and resonances are given downfield (δ) from the internal standard Me_4Si (δ 0.00). Microanalyses were performed by the Analytical Laboratory, Chemistry Department, University of California, Berkeley.

(*R*)-1-Benzyl-5-[4-(2-methyl-1,3-dioxolan-2-yl)-1-(benzyloxycarbonyl)butylidene]proline *tert*-Butyl Ester (4). Triflate 3 (21.3 g, 50 mmol) was dissolved in CH_3CN (35 mL), and (D)-*tert*-butyl N-benzylthiopyroglutamate (2; 12.0 g, 41.1 mmol) was added. The mixture was stirred at room temperature under Ar overnight. After dilution with dry CH_2Cl_2 (350 mL), the solution was cooled to −20 °C, triphenylphosphine (12.9 g, 49.3 mmol) was added, the mixture was stirred for 45 min, and then N-methylpiperidine (6.4 mL, 5.2 g, 54.5 mmol) was added via syringe at a rate of 0.39 mL min^{-1}. Stirring was continued for 6 h, allowing the bath temperature finally to reach 0 °C. The solution was then washed with 1 M KH_2PO_4 (2 × 150 mL) and saturated $NaHCO_3$ (150 mL). Drying, filtering, and evaporating gave crude product, which was purified by chromatography, eluting with CH_2Cl and then 5% EtOAc in CH_2Cl_2 to give 18.4 g, 84% yield, of 4.

(2*R*)-*cis*-5-[4-(2-Methyl-1,3-dioxolan-2-yl)butyl]proline *tert*-Butyl Ester (6). A solution of vinylogous carbamate 4 (16.1 g, 30 mmol) in MeOH (200 mL) was degassed (N_2), and 10% Pd/C (9.6 g) and ammonium formate (18.9 g, 300 mmol) were added. The mixture was vigorously refluxed for 150 min, filtered hot, and evaporated. The residue was taken into 10% Na_2CO_3 (50 mL) and extracted with CH_2Cl_2 (3 × 50 mL). Drying, filtering, and evaporating gave a pale yellow oil that was purified by chromatography, eluting with a gradient of MeOH in CH_2Cl_2 (0–5%) to give 6 (6.3 g, 67% yield).

(2*R*)-*cis*-1-Benzyl-5-[4-(methyl-1,3-dioxolan-2-yl)butyl]proline *tert*-Butyl Ester (8). A solution of pyrrolidine 6 (11.5 g, 37 mmol), K_2CO_3 (15.2 g, 111 mmol), and benzyl bromide (6.6 g, 39 mmol) in CH_3CN (50 mL) was stirred under N_2 at room temperature for 12 h. The reaction mixture was then diluted with water (100 mL) and extracted with $CHCl_3$ (3 × 150 mL). The organic extracts were dried, filtered, and evaporated. Purification of the crude pale yellow oil by MPLC (SiO_2, 1/4 EtOAc/isooctane + 0.2% Et_3N) gave pure 8 (13.7 g, 93% yield).

(30) Zachariesen, W. H. *Acta Crystallogr.* **1963**, *16*, 1139.

(2*R*)-*cis*-1-Benzyl-5-(5-oxohexyl)proline (9) was prepared as reported.[6] After repeated azeotropic removal of residual solvents with toluene, a pale yellow semisolid mass was obtained. Trituration with ether and recrystallization from toluene afforded pure 9 hemihydrate in quantitative yield: mp 86–87 °C; ^{13}C NMR δ 23.0, 25.5, 28.0, 29.9, 30.0, 32.6, 42.9, 57.5, 66.7, 67.9, 129.1, 130.1, 132.1, 172.0, 208.5. Anal. ($C_{18}H_{25}NO_3$) C, H, N.

(5*R*)-1-Benzyl-2-cyano-5-(5-oxohexyl)pyrrolidine (12). Amino acid 9 (150 mg, 0.5 mmol) was dissolved in $POCl_3$ (0.5 mL, 5 mmol), and the mixture was heated at 90 °C until gas evolution ceased (15 min). The mixture was then allowed to cool, and volatiles were removed in vacuo. The crude iminium salt 11 was dissolved in CH_2Cl_2 (7 mL) and treated for 30 min at room temperature with aqueous KCN (330 mg, 5 mmol in 2 mL of H_2O), buffering (NaOAc) the aqueous layer to pH 4. Basification with 10% aqueous Na_2CO_3, extraction with CH_2Cl_2, drying, filtration, and evaporation gave a pale brown oil. Column chromatography (CH_2Cl_2) provided pure 12 as an approximately 1/3 mixture of cis–trans isomers (118 mg, 84% yield): IR 2210, 1690 cm^{-1}; ^1H NMR δ 1.2–2.2 (10 H, m), 2.11, 2.13 (3 H, s, each 1/3), 2.36–2.47 (2 H, m), 2.65–2.80 (1 H, m), 3.43 and 4.05 (d, benzylic major isomer), 3.87 (AB quartet, benzylic, minor isomer), 3.4–3.5 and 3.70 (dd, 1 H), 7.2–7.4 (m, 5 H); ^{13}C NMR δ (major isomer) 23.9, 25.0, 27.4, 28.3, 29.8, 33.3, 43.4, 53.4, 53.7, 61.1, 117.6, 127.3, 128.4, 128.7, 137.8, 208.7. Anal. ($C_{18}H_{24}N_2O$) C, H, N.

(1*R*)-2-Acetyl-9-benzyl-9-azabicyclo[4.2.1]nonane (11). A. From 9. Amino acid 9 (4.15 g, 13.7 mmol) was dissolved in $POCl_3$ (20.7 g, 135 mmol) and heated to 90 °C for 15 min. The solution was then evaporated and the residue dissolved in methanol (150 mL) presaturated with HCl gas. The resulting solution was heated in an oil bath at 55 °C under N_2 for 20 h. Then, most of the methanol was evaporated and the residue was taken into CH_2Cl_2 (150 mL). An aqueous solution of KCN (4.5 g, 70 mmol, in 50 mL of H_2O) was added, the pH was adjusted to 4 with NaOAc, and the two-phase mixture was stirred vigorously for 30 min. The aqueous phase was basified to pH 9 with 10% aqueous Na_2CO_3 and extracted with CH_2Cl_2 (3 × 75 mL). The combined organic solutions were dried, filtered, and evaporated to a pale brown oil (3.48 g). Rapid column chromatography (CH_2Cl_2 then 5% MeOH in CH_2Cl_2) provided 11 (2.48 g, 70%) and 12 (0.26 g, 9%).

B. From (12). Amino nitrile 12 (90 mg, 0.32 mmol) was dissolved in methanol (15 mL) saturated with HCl gas, and the solution was heated at 60 °C for 18 h. The solvent was evaporated and the residue partitioned between saturated aqueous $NaHCO_3$ and CH_2Cl_2. Drying, filtration, and evaporation of the organic phase provided 75 mg, 93% yield, of crude product which was homogeneous by TLC. Chromatography as above gave pure 11 (63 mg, 78% yield).

(1*R*)-2-Acetyl-9-(*tert*-butoxycarbonyl)-9-azabicyclo[4.2.1]2-nonene (16). Boc-dihydroanatoxin-a[6] (14; 1.26 g, 4.7 mmol) in 5 mL of THF was added to a suspension of KH (1.00 g, 20%, 5 mmol) in 10 mL of THF. The mixture was stirred at 35 °C for 3 h and cooled in ice, and triethylamine (1.3 mL, 9.4 mmol) and Me_3Si–Cl (0.8 mL, 0.67 g, 6.2 mmol) were added. After stirring for 1 h at 0 °C, the solution was diluted with CH_2Cl_2 (30 mL) and rapidly washed with 0.01 M pH 7 phosphate buffer. Brief drying, filtration, evaporation, and final drying gave crude 15. The silyl enol ether 15, $Pd(OAc)_2$ (1.0 g, 4.7 mmol), and triethylamine (1.0 mL, 0.74 g, 7.4 mmol) in CH_3CN (30 mL) were stirred for 36 h. Filtration, evaporation, and purification as described previously gave 14 (0.56 g) and 16 (0.36 g, 52% yield based on recovered 14).

(1*R*)-2-Acetyl-9-azabicyclo[4.2.1]2-nonene Hydrochloride (1·HCl). As described previously[6] 1·HCl was prepared from 16 in quantitative yield. The glassy hydrochloride was redissolved in absolute ethanol, and the solution was evaporated. The residue was then freed of excess water by repeated azeotropic distillation with benzene. Final drying under high vacuum overnight, dissolving the glassy solid in 3% methanol in diethyl ether, and standing at 4 °C for 7 days gave pure 1·HCl as a colorless solid: mp 152.5–153 °C; ^{13}C NMR (free base) δ 24.8, 25.4, 30.1, 32.8, 33.2, 53.9, 57.6, 143.2, 152.1, 198.5. The 500-MHz ^1H NMR spectra assignments for 1 and 1·HCl are given in Table III.

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Registry No. 1, 64285-06-9; 1·HCl, 64314-16-5; 2, 90741-32-5; 3, 96929-76-9; 4, 96998-26-4; 6, 90741-62-1; 8, 90822-44-9; 9, 90741-47-2; 10, 96929-77-0; 11, 96929-78-1; 12 (isomer 1), 96929-79-2; 12 (isomer 2), 96997-81-8; 14, 96997-82-9; 15, 96929-80-5; 16, 90741-53-0.

Supplementary Material Available: The X-ray crystallographic determination of 1·HCl, listings of fractional atomic coordinates with their estimated standard deviations, temperature factors, intramolecular distances and angles, least-squares planes, and observed and calculated structure factors, and force field calculations giving complete coordinates for structures A–D (13 pages). Ordering information is given on any current masthead page.

Effects of Charge, Volume, and Surface on Binding of Inhibitor and Substrate Moieties to Acetylcholinesterase

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Reversible inhibitors for acetylcholinesterase, AcChE, have been studied. Sterically similar alcohols with tetrasubstituted uncharged β groups, $(CH_3)_3SiCH_2CH_2OH$ (I), $(CH_3)_3CCH_2CH_2OH$ (IA), and $CH_3S(O_2)CH_2CH_2OH$ (VII), bind similarly, K_I = 3–9 mM, and each binds similarly to its acetate substrate; cationic analogues, $(CH_3)_3N^+CH_2CH_2OH$ (IB) and $(CH_3)_2S^+CH_2CH_2OH$ (II), bind similarly to each other, K_I = 0.4 mM, similar to K_m values of their acetate substrates, and more strongly than the uncharged alcohols by ~1.5 kcal/mol. In comparisons of VII with $CH_3SO_2CH_3$, II with $(CH_3)_3S^+$, and IB with $(CH_3)_4N^+$, hydroxyethyl leads to more favorable binding than methyl by ~0.8 kcal/mol, despite lower hydrophobicity. Two hydrophobic methyl groups, in comparison of IA with butanol, and two hydrophilic sulfone O atoms, in comparison of VII with 2-(methylthio)ethanol, increase binding similarly, by 1.0 kcal/mol. Conversion of $(CH_3)_3S^+$ to $(CH_3)_3S^+O$ also improves binding. However, $(CH_3)_3N^+O^-$ does not bind to AcChE, and conversion of 1-(dimethylammonio)-4-pentanone and 2-(dimethylammonio)ethyl acetate to their *N*-oxides, changes of $\equiv N^+H$ to $\equiv N^+—O^-$, decreases binding by 1.5 kcal/mol. Although the $-COCH_3$ group in esters with well-binding β substituents makes essentially no contribution to binding over that of the alcohols, in esters with weakly bound β substituents, $(CH_3)_2N^+(O^-)$, $CH_3N^+H_2$, $CH_3S(O)$, CH_3CH_2, and CH_3S binding is dominated by the ester $-COCH_3$ group, with values of $K_m \sim 16$ mM.

Acetylcholinesterase, AcChE, hydrolyzes ethyl acetates, $X-CH_2CH_2OCOCH_3$, with cationic,[1] nonpolar,[2] and uncharged polar[3] β substituents, X, of varied structure. Enzymic reactivity normalized for effect of β substituents on intrinsic alkaline hydrolytic reactivity, $(k_{cat}/K_m)_n$, for cationic and neutral substrates with X = $(CH_3)_3C$, $(CH_3)_3N^+$, $(CH_3)_2CH$, $(CH_3)_2S^+$, CH_3CH_2, Br, Cl and H was correlated with calculated refraction volumes, MR, while with X = CH_3S, $CH_3S(O)$, $(CH_3)_2N^+O^-$ and $CH_3S(O_2)$ reactivity was *lower* than consistent with MR by factors of 5–40.[4] Normalized reactivity of substrates with β substituents Cl, Br, CH_3S, CH_3CH_2, $(CH_3)_2CH$, $(CH_3)_3C$, and $(CH_3)_3Si$ correlated with hydrophobicity, π, but the cationic and dipolar substituents, $(CH_3)_3N^+$, $(CH_3)_2S^+$, $CH_3S(O_2)$, $CH_3S(O)$, and $(CH_3)_2N^+(O)^-$, led to reactivity greater than consistent with a relation to π by factors of 7–400, with the cationic substituents showing the greatest discrepancies.[4] Thus, it appeared that there is a more general and relevant correlation of reactivity with volume than with hydrophobicity, π, i.e. favorable lipid to water solubility ratio, and that maximum reactivity, correlated with volume, may depend on presence of a hydrophobic surface.[4] This was consistent with the view that the binding subsite for the β substituent may be termed *trimethyl* rather than *anionic*, apparently complementary to the hydrocarbon surface of analogous cationic and uncharged branched β substituents.[5] Support for the uncharged character of this subsite was seen in the equal effectiveness of α-bromopinacolone, $(CH_3)_3CCOCH_2Br$, in irreversible inhibition of hydrolysis of acetylcholine and its uncharged carbon analogue, 3,3-dimethylbutyl acetate.[6]

Cationic charge increases binding of cationic as compared with isosteric uncharged reversible inhibitors structurally related to acetylcholine, by about a factor of 10,[7] as the isoelectric point of the enzyme, ~5,[8] leads to multiple nonspecific anionic charges in the region of the active site[9] at the higher pH, 7–8, at which the enzyme acts. That these sterically similar cationic and uncharged pairs inhibit acetylcholine and its uncharged analogue equally indicates that a single subsite is involved in the binding

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