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Modified γ-Cyclodextrins and Their Rocuronium Complexes

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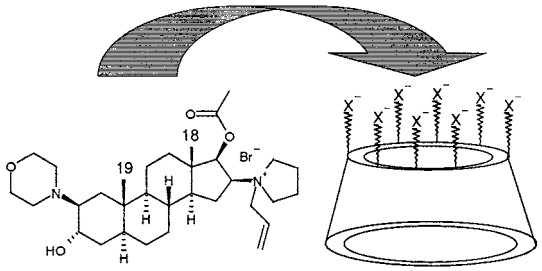
ABSTRACT



A series of per-6-substituted cyclodextrin derivatives was synthesized as synthetic host molecules for rocuronium, a steroidal muscle relaxant. By forming host–guest complexes with rocuronium, these cyclodextrin derivatives reverse the muscle relaxation induced by rocuronium in vitro and in vivo. The isothermal microcalorimetry data are consistent with the biological data supporting the encapsulation mechanism of action. Binary and biphasic complexes are reported with NMR experiments clearly showing free and bound rocuronium.

The reversal of neuromuscular block at the end of surgery is often necessary to speed up the recovery of a patient's muscle function and to prevent residual neuromuscular block.

All current clinically used reversal agents, such as neostigmine and edrophonium, exert their activity by inhibiting acetylcholine esterase (AChE), to increase the levels of acetylcholine (ACh) at the neuromuscular junction.[1] Unfortunately this mechanism of action has the intrinsic disadvantage of nonselective potentiation of muscarinic acetylcholine receptors (mAChR), leading to cardiovascular side-effects.

We have therefore been interested in developing compounds that operate by an alternative mode of action and overcome the above drawbacks and limitations of AChE inhibitors.[2,3] Chemical encapsulation of neuromuscular blocking agents (NMBAs) by a host molecule such as a cyclodextrin (CD) is one of these mechanisms that we have been actively pursuing.[4,5] Previously we have described design, synthesis, and structure−activity relationships of a series of derivatized CDs[5,6] as potential host molecules to reverse the effect of the neuromuscular blocking agent rocuronium, the

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most widely used NMBA in anaesthesia. It was shown that a cavity diameter of γ-CD (7.5−8.3 Å) and negatively charged carboxylate groups at the rim of the CD cavity were necessary for strong binding. The negative charge contributes to binding in two ways: (1) by forming electrostatic interactions with the quaternary nitrogen of rocuronium, and (2) holding the entrance open by maintaining the preorganization of the host because of electrostatic repulsion.

In this paper, we report a further series of 6-deoxy-6-substituted γ-CDs containing carboxylate functionality, and their reversal activity toward rocuronium-induced neuromuscular block. The relationship between rocuronium reversal activity and the thermodynamic properties of the inclusion complexes is discussed. Two CD/rocuronium complexes were examined in more detail using ^{1}H NMR and microcalorimetry.[7]

In this study three different series of CD hosts for rocuronium are compared. The 6-per-thiolated γ-CD derivatives (alkyl and thiophenolic) were prepared according to methods previously described,[5] and the 6-per-phenolic γ-CD derivatives were prepared[8] from the 2,3-per-acetyloxy-γ-cyclodextrin[9] via the 6-per-triflate[10] with an appropriate phenol.

The correlation between CD-rocuronium complex stability (highlighted by K_a) and the reversal potency (in vitro and in vivo) is good (Table 1). This correlation adds support for the encapsulation mechanism of action of these compounds. The reversal potencies of these compounds are shown to be related to the chain length (Table 1, **2**−**6**). Increasing the chain length generally lowers the reversal potency both in vitro and in vivo, with a two-carbon chain linker, **3,** being optimal. This is in accordance with the isothermal microcalorimetry (ITC) data, which shows **3** to have the highest association constant (K_a 1.80 × 10^7 M^{-1}). This complex has a negative ΔH and positive ΔS value, which indicates that the complexation is both enthalpically and entropically favored. In fact, this is one of the most stable complexes of a CD with an organic guest reported in the literature. A greatly extended spacer group between the CD backbone and terminal carboxyl group **6** decreased reversal potency. The lower potency of this compound could be due to a greater stability of self-inclusion (hydrophobic collapse) of the more flexible side chains.

Comparison of **2**−**6** with the aromatic substituents **7**−**11** show that the short alkyl chains exhibit association constants larger than those of the benzoic and phenylacetic acid derivatives. This strong binding is reflected in their superior biological activity. The oxygen-linked benzoic and phenylacetic acid analogues, **12**−**16**, are generally less strongly bound than the thio analogues. It is interesting to note that binding is endothermic in the ether-linked compounds **12**−**16** (entropically driven) but exothermic in the case of corresponding thioethers **7**−**11** (enthalpically and entropically driven). The enthalpic contribution is believed to derive from the formation of noncovalent bonds (hydrophobic interactions) in the binding site. The favorable entropy term is based on the expulsion of the significant number of water molecules from the large γ-CD cavity. CD **9** is exceptional in that it demonstrates biphasic binding in ITC (Figure 3b), which might indicate multiple binding sites or, alternatively, more complicated binding mechanism involving ligand-induced aggregation to form higher-stiochiometry complexes. Within both the benzoic and phenylacetic acid series, para substitution gives the stronger binding and meta the weakest. Again, these trends are reflected in the reversal activity both in vitro and in vivo.

The complex with the strongest binding **3**/rocuronium and the complex showing the uncharacteristic biphasic binding **9**/rocuronium were explored in more detail by ^{1}H NMR.

An NMR study on the CD complexes with rocuronium has been reported previously.[11] The strong binding between the CDs and rocuronium was directly observed by ^{1}H NMR. Room temperature ^{1}H NMR spectra of **3**/rocuronium mixtures show the system to be in slow exchange on the NMR time scale, and so both free **3** and free rocuronium and the **3**:rocuronium complex could be observed at different ratios (Figure 1). The 1:2 spectrum (Figure 1b) displays the doubling of signals that results from observing the steroid axial methyl signals (18-CH_3 and 19-CH_3) in two different environments. In this solution the steroid is present at double

(7) All ^{1}H NMR experiments were performed at 400 MHz on a Bruker DRX spectrometer at 303 K. Samples were dissolved in D_2O at pH 7.5. For titrations, samples typically contained 1.0 mM rocuronium and CD in the range of 0.2−5.0 mM and vice versa. Microcalorimetry experiments[6] were performed using MicroCal ITC. Experiments were done at 298 K with sample concentrations of 0.01−0.1 mM CD and 0.2−2.0 mM rocuronium.

(8) **CD-Ether Formation As Exemplified by the Preparation of 6-Per-(4-carboxyphenyl)-γ-cyclodextrin 14.** 2,3-Per-acetyloxy-γ-cyclodextrin[9] was converted to the 6-per-triflate derivative in DCM/pyridine by reaction with triflic anhydride. The 6-per-triflate-γ-cyclodextrin (1.37 g, 0.45 mmol) and methyl-4-hydroxybenzoate (0.91 g, 6.0 mmol) were dissolved in acetone (20 mL) and then sonicated at 30 °C for 2.5 h in the presence of potassium carbonate (0.84 g, 6.1 mmol). Solids were removed by filtration before flash chromatography on silica gel, eluting with EtOAc. The residue was treated with 4 M NaOH (40 mL)/EtOH (30 mL) for 10 min at 50 °C, before evaporation of volatiles and heating for 2 h. The solution was then dialyzed (MWCO 1000) for 6 h. Concentration of the aqueous solution to low volume was followed by precipitation with acetone. The residue was sonicated for 2 h in a 1:1 mixture of MeOH/acetone and filtered to give 6-per-(4-carboxyphenyl)-γ-cyclodextrin sodium salt as a white solid (720 mg). ^{1}H NMR (D_2O, ppm) 7.75 (16H, d, J = 8.7 Hz), 6.79 (16H, d, J = 8.7 Hz), 5.16 (8H, d, J = 3.6 Hz), 4.03−4.06 (24H, m), 3.84 (16H, m), 3.63 (8H, dd, J = 3.7 and 9.7 Hz); m/z 2256.9 (M − 8Na + 7H)$^-$. **Data for 12.** ^{1}H NMR (D_2O, ppm) 7.26 (8H, d, J = 8.0 Hz), 7.24 (8H, d, J = 2.0 Hz), 7.12 (8H, t, J = 8.0 Hz), 6.75 (8H, dd, J = 8.0 and 2.0 Hz), 5.08 (8H, d, J = 3.5 Hz), 4.00−3.86 (24H, m), 3.81 (8H, d, J = 10.0 Hz), 3.74 (8H, t, J = 10.0 Hz), 3.50 (8H, dd, J = 10.0 and 4.0 Hz); m/z 2256.2 (M − 8Na + 7H)$^-$. **Data for 13.** ^{1}H NMR (D_2O, ppm) 7.22 (8H, dd, J = 7.5 and 2.0 Hz), 7.17 (8H, dt, J = 7.5 and 2.0 Hz), 6.81 (8H, t, J = 7.5 Hz), 6.77 (8H, t, J = 8.0 Hz), 5.04 (8H, d, J = 4.0 Hz), 4.00−3.70 (32H, m), 3.54 (8H, dd, J = 9.5 and 3.5 Hz); m/z 2256.2 (M − 8Na + 7H)$^-$. **Data for 15.** ^{1}H NMR (D_2O, ppm) 7.17 (8H, t, J = 8.0 Hz), 7.09 (8H, d, J = 7.5 Hz), 6.90 (8H, t, J = 7.5 Hz), 6.63 (8H, d, J = 8.0 Hz), 5.07 (8H, d, J = 4.0 Hz), 3.97−3.80 (24H, m), 3.78 and 3.65 (16H, dd, J = 53.7 and 11.0 Hz), 3.55 (8H, dd, J = 10.0 and 3.5 Hz), 3.50 (16H, dd, J = 20.6 and 16.6 Hz); m/z 2369.2 (M − 8Na + 7H)$^-$. **Data for 16.** ^{1}H NMR (D_2O, ppm) 7.10 (16H, d, J = 8.6 Hz), 6.78 (16H, d, J = 8.6 Hz), 5.15 (8H, d, J = 4.0 Hz), 4.07 and 3.97 (16H, dd, J = 9.6 and 50.9 Hz), 4.02 (8H, t, J = 9.1 Hz), 4.01 (8H, m), 3.81 (8H, t, J = 9.6 Hz), 3.61(8H, dd, J = 10.1 and 4.0 Hz), 3.34 (16H, dd, J = 19.6 and 15.1 Hz); m/z 2269.0 (M − 8Na + 7H)$^-$.

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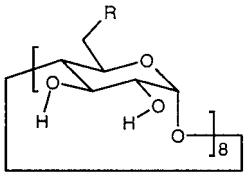
Table 1. Structures and Reversal Activities of Per-6-thiolated CDs against Rocuronium-Induced Neuromuscular Block



		isothermal microcalorimetry				in vitro[a] reversal activity		in vivo[b] reversal activity	
cmpd	R	K_a (mol^{-1})[c]	ΔH (kJ/m ol)	ΔG (kJ/mol)	ΔS (J/mol/K)	EC_{50}, μM	max reversal, % (concn, μM)	ED_{50}, μmol/kg	max reversal, % (dose, μmol/kg)
1	OH[f]	1.32×10^4	4.2	−23.51	92.75	34.6	94.1 ± 2.0 (144)	4.0	104.7 ± 8.6 (47)
2	SCH_2CO_2Na[g]	5.41×10^6	−24.76	−38.54	46.64	1.2	93.8 ± 2.7 (3.6)	0.10	103.3 ± 4.3 (0.5)
3	$S(CH_2)_2CO_2Na$[g]	1.80×10^7	−28.9	−41.53	42.81	1.2	95.1 ± 2.3 (3.6)	0.03	92.5 ± 5.3 (0.3)
4	$S(CH_2)_3CO_2Na$[g]	6.10×10^6	−20.92	−38.84	60.42	1.4	98.5 ± 4.5 (3.6)	0.06	93.4 ± 10.6 (0.3)
5	$S(CH_2)_4CO_2Na$[g]	2.30×10^6	−17.43	−36.46	64.13	1.8	98.9 ± 5.2 (5.4)	0.07	99.0 ± 3.5 (0.3)
6	$S(CH_2)_5CO_2Na$[g]	1.40×10^5	−9.79	−29.36	67.15	7.0	81.7 ± 12.6 (12.6)	0.74	78.4 ± 7.7 (2.5)
7	*o*-*S*-$PhCO_2Na$[g]	9.10×10^3	−3.17	−22.59	65.17	226.7	42.3 ± 13.8 (216)	n.t.[d]	n.t.[d]
8	*m*-*S*-$PhCO_2Na$[g]	3.24×10^5	−7.19	−31.44	81.37	3.3	95.7 ± 2.3 (7.2)	0.28	102.0 ± 5.7 (1.3)
9	*p*-*S*-$PhCO_2Na$[g,h]	$\sim 2.0 \times 10^6$; ~ 1000	~4; ~−50	~−36; ~−17	~130; ~−110	1.0	97.0 ± 4.2 (2.2)	0.12	97.6 ± 6.3 (1.4)
10	*m*-*S*-$PhCH_2CO_2Na$[g]	5.75×10^5	−18.66	−32.86	47.66	1.7	99.1 ± 5.2 (7.2)	0.53	104.6 ± 0.8 (2)
11	*p*-*S*-$PhCH_2CO_2Na$[g]	2.46×10^6	−14.09	−36.46	75.14	1.4	98.9 ± 1.1 (2.9)	0.14	103.8 ± 2.8 (1.6)
12	*o*-*O*-$PhCO_2Na$	3.17×10^4	0.74	−25.68	88.51		0.0 ± 0.0 (18.0)		22.2[e] (16.0)
13	*m*-*O*-$PhCO_2Na$	5.16×10^4	27.91	−26.88	173.76	5.0	94.6 ± 1.9 (16.2)	2.3	81.9 ± 9.9 (10.5)
14	*p*-*O*-$PhCO_2Na$	2.87×10^5	37.67	−31.14	230.81	1.0	100.2 ± 1.9 (0.3)	0.32	100.5 ± 6.5 (3.5)
15	*o*-*O*-$PhCH_2CO_2Na$	3.50×10^4	0.99	−25.92	90.12	5.0	94.6 ± 1.9 (16.2)	-	8.4[e] (8.0)
16	*p*-*O*-$PhCH_2CO_2Na$	2.33×10^5	20.21	−30.62	170.54	1.5	88.3 ± 7.2 (3.6)	0.3	96.2 ± 10.9 (1.6)

[a] Versus ~90% block by rocuronium (isolated mouse hemidiaphragm). Data are presented as means ± SEM of at least 4 independent experiments. The concentration of rocuronium in the organ bath was 3.6 μM, which produced ~90% reduction of the twitch height. EC_{50} = concentration that produces 50% recovery of muscle twitch compared with prereversal twitch height. Max reversal % = maximum twitch recovery achieved with highest concentration tested in bracket. [b] Versus ~90% block by rocuronium (i.v., guinea pigs). Data are presented as means ± SEM of at least 2 independent experiments or a single experiment when the potency was of low interests. An average of 90% (80−97%) neuromuscular block was achieved by continuous i.v. infusion (~10 nmol/kg/min) of rocuronium and applying cumulative doses of CD. ED_{50} = dose (i.v.) that produces 50% recovery of muscle twitch compared with preblock twitch height. Max reversal % = maximum twitch recovery achieved with highest dose tested in bracket. [c] Stoichiometry of complexes 1:1. [d] Not tested. [e] n = 1. [f] Commercial product. [g] Synthesis has been reported previously.[5] [h] Fit assuming a two-site binding model.

the concentration of the CD, and the integrated peak ratios (bound versus free) are consistent with very strong binding and formation of a 1:1 complex. This indicates the formation of a strong and stable complex under these conditions. The association constant of the complex could not be determined using the ^{1}H NMR chemical shift differences between the complexed and free rocuronium signals. ^{1}H NMR can only measure association constants up to ca. 10^4 M^{-1}, and since the complex has already been observed to have a binding constant of 1.8×10^7 M^{-1} by ITC, it was not feasible to use NMR.

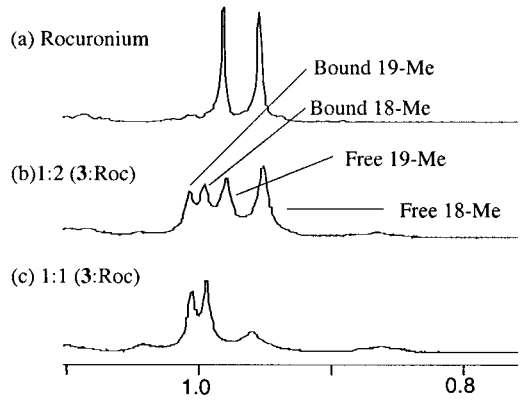


Figure 1. ^{1}H NMR spectra of **3** and rocuronium (Roc) at different molar ratios.

CD **9** forms a host−guest complex with rocuronium as evidenced by ITC measurements and appears to have a biphasic binding to rocuronium (Figure 3b), with the first step endothermic. Endothermic complexation with rocuronium is also observed in the ether analogue of CD **9**. Although it is possible to model this biphasic behavior in terms of two independent binding sites (data given in Table 1) it is also possible that addition of rocuronium induces the formation of a 2:1 CD sandwich complex encapsulating the rocuronium host. Either model may explain why the ^{1}H NMR spectrum of **9** is extremely broad at room temperature and sharpens on complexation with rocuronium. The ^{1}H NMR spectrum of the 1:1 mixture of **9** and rocuronium (Figure

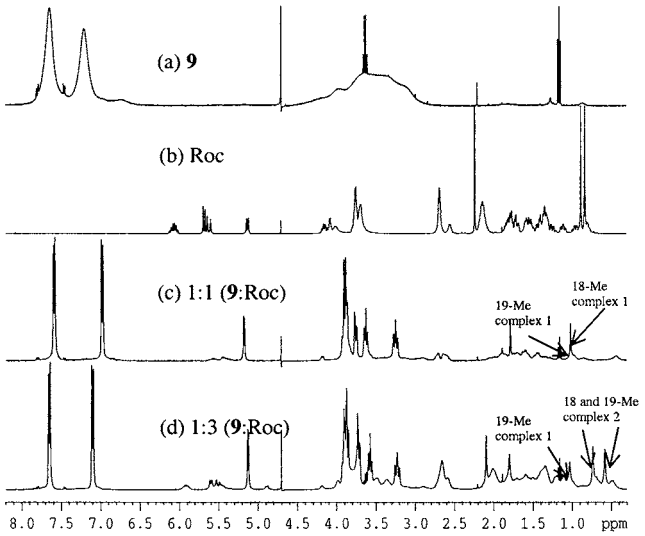


Figure 2. ^{1}H NMR spectra of **9** and rocuronium (Roc) at different molar ratios.

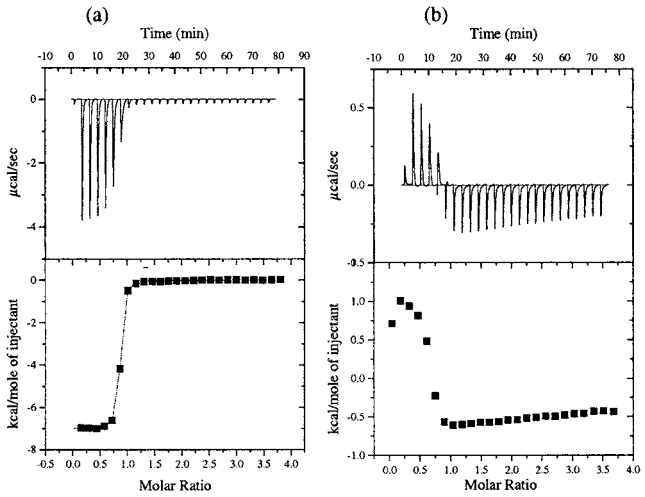


Figure 3. ITC curves for (a) **3**/rocuronium complex and (b) **9**/rocuronium complex.

2c) shows that the steroid resonances are somewhat broad, notably the 18-CH_3. This is due to the fact the rocuronium is in slow exchange between the bound and free states. Spectrum (d) in Figure 2 shows that with an excess of rocuronium a new set of sharp steroid resonances appear (the new resonances are most obvious for the axial methyls at 0.6−1.0 ppm). The two sets of signals were initially thought to be from free and bound rocuronium as was seen for the **3**/rocuronium complex.

The association constant (K_a) for the complex has been measured at ∼2.0 × 10^6 M^{-1} by ITC. However, assuming the two-site binding model is correct, ITC also indicates that there is a K_a of ∼1000 M^{-1} for a second molecule of rocuronium binding to **9**. It is quite clear from Figure 2 that signals from both rocuronium and **9** are changing as the ratio of the steroid is increased. Therefore, the second set of sharp signals can be used to determine the association constant of this secondary binding. The K_a was determined by NMR by a titration from 1:1 to 1:5 molar ratio of **9** to rocuronium, respectively. The chemical shift of the 19-CH_3 signal in rocuronium changed significantly (0.73−0.82 ppm) during the titration, and the resulting K_a of 1340 M^{-1} was determined by a curve fitting method.[12] The NMR result is in good agreement with the value determined by ITC (1790 M^{-1}) and so confirms the complexation of a second molecule of rocuronium by **9**.

In summary we have synthesized CD-based host molecules that complex with rocuronium with high affinity as shown by ITC and ^{1}H NMR. Particularly interesting is the difference in the type of binding between thio-linked (exothermic) and oxygen-linked (endothermic) CDs. The thio-linked CD complexation is driven by the hydrophobic interaction and the release of water, whereas complexation with oxygen-linked CDs is dominated by the release of even greater amounts of water from the extended CD cavity. Uncharacteristic biphasic binding has been shown for one complex, and both the ITC and NMR experiments are in good agreement for the association constant of the second mode of binding.

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