Solubilization and Stabilization of a Benzylpenicillin Chemical Delivery System by 2-Hydroxypropyl-β-cyclodextrin

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A dihydropyridine ↔ pyridinium salt redox carrier-based chemical delivery system for benzylpenicillin (1) was complexed with 2-hydroxypropyl-β-cyclodextrin (HPCD). The solubility of the lipophilic 1, which is incompatible with aqueous formulations, was dramatically increased and showed a linear dependency on the HPCD concentration. The degree of incorporation was 20 mg of 1 per g of complex. The stability study of 1 in various pH buffers indicated the base-catalyzed hydrolysis of the acyloxyalkyl linkage and the hydration of the 5,6 double bond of the dihydropyridine as the main degradation processes. The overall loss of 1, which follows first-order kinetics, was not influenced by changes in ionic strength and elimination of oxygen from the reaction medium. The HPCD complex of 1, which has a stability constant of 720–940 M^{-1}, stabilized the chemical delivery system. The influence of the temperature on the stability of 1 is also discussed.

KEY WORDS: benzylpenicillin; chemical delivery systems; 2-hydroxypropyl-β-cyclodextrin.

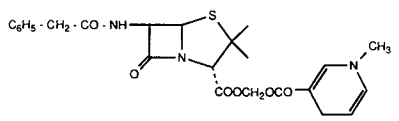
INTRODUCTION

β-Lactam antibiotics have poor access to the central nervous system (CNS) across the protective lipoidal blood–brain barrier (BBB) because of their polar, hydrophilic nature (1–3); additionally, they are rapidly eliminated from the CNS into blood by active transport mechanisms (4–6). However, penicillins and cephalosporins are widely used in the management of bacterial infections of the brain, as they have a low toxicity and low minimum inhibitory concentration (MIC) (7). Benzylpenicillin, for example, is still a drug of choice in the treatment not only of bacterial meningitis, which causes an increased permeability of the BBB to the drug (1,2,8), but also of other infections of the brain, such as AIDS-related brain syphilis (9–11), brain abscesses (12,13), Lyme disease, etc.; in these cases, the access of the antibiotic to the CNS is very limited, the achieved cerebrospinal fluid (CSF)/plasma ratios of the drug concentration being only 1–2%. High dosages of antibiotic (up to 14 g daily) (14) or the simultaneous administration of probenicid (15), a drug which inhibits the excretion of the penicillin, are required in order to obtain active CNS concentrations, practices which may have limitations and undesired side effects (16). A recently developed approach, the dihydropyridine ⟷ pyridinium salt-type redox carrier-based chemical delivery systems (17–19), seems to offer a solution to these problems when applied to penicillins. The chemical delivery systems allow for a selective brain uptake of drugs, a prolonged presence of the drug in the CNS and a rapid peripheral elimination. Various types of chemical delivery systems were synthesized for benzylpenicillin (20) and subjected to a "screening" process based on the results of extended *in vitro* and *in vivo* studies (20–22). One of these derivatives (**1**, Scheme I) manifested CNS selectivity, sustained presence in active concentrations in the CSF and brain and a lack of toxic side effects when administered to rats, rabbits, and dogs. Accordingly **1** was considered a justified candidate for more detailed investigation, including clinical trials (23).

In spite of the enumerated advantages, a poor solubility in water and a reduced chemical stability could limit the chances of **1** to become a drug of practical use. The possibility to overcome these disadvantages has been investigated. It is known that modified cyclodextrins, such as the highly water-soluble 2-hydroxypropyl-β-cyclodextrin (HPCD), can encapsulate numerous compounds at the molecular level, conferring on them new physicochemical and pharmacotechnical properties, including increased solubility and bioavailability, greater stability, and reduced incidence of side effects (24–27). The purpose of this study was to examine some of these effects (stability, solubility in water) of HPCD on **1**. Positive results could offer the possibility of using the reversible water-soluble **1**–HPCD inclusion complexes as parenterally appropriate aqueous dosage forms.

MATERIALS AND METHODS

[[(1,4-Dihydro-1-methyl-3-pyridinyl)carbonyl]oxy]-methyl[2*S*-(2α,5α,6β)]-3,3-dimethyl-7-oxo-7-[(phenylacetyl) amino]-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylate (**1**) was prepared by a modification of a previously described method (20). Briefly, benzylpenicillin K salt was reacted in dimethylformamide with chloromethyl nicotinate (synthesized from nicotinic acid and chloromethyl chlorosulfate under phase-transfer conditions) and the resulting nicotinate, obtained with a yield of 80%, was quaternized with methyl iodide or methyl-*p*-toluenesulfonate in refluxing ethyl acetate; the quaternary salt-type derivatives, which resulted with 90–95% yields, were then regioselectively reduced to the 1,4-dihydropyridine derivative with sodium dithionite (yield, 70%). 2-Hydroxypropyl-β-cyclodextrin (HPCD) was synthesized from β-cyclodextrin and propylene oxide by a



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Scheme I

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known procedure (27). All other chemicals were commercially available and of reagent grade.

Solubility Studies

The solubility of 1 in HPCD was determined at various cyclodextrin concentrations. An excess of 1 was added to solutions of HPCD in deionized water (Barnstad, Nanopure II Ultrapure Water System); the resultant suspensions were sonicated in an ultrasonic bath (Kerry, England) for 30 min and placed in a 30.0 ± 0.1°C constant-temperature water bath (Tempette, Techne, England). After equilibrium was attained (30 min), an aliquot was filtered through a 0.45-μm membrane filter unit (Millex—HV_4, Millipore, Milford, MA) and analyzed by HPLC.

Cosolvent Technique for Solubilization

A solution of 1 g of 1 in 25 ml of absolute ethanol was sonicated for 15 min with a solution of 25 g of HPCD in 50 ml of water at pH 6.5–7. The solvents were evaporated *in vacuo* (rotavapor) and the glassy residue reconstituted in 50 ml ultrapure water. The solution was filtered through a 0.45-μm hydrophilic membrane, lyophilized (Labconco Freeze Dryer Model 18), and passed through a 60-mesh (250-μm) sieve. The amount of 1 incorporated into the complex was determined by HPLC.

Analytical Methodology

The studied compound 1 was quantitated by a reversed-phase high-performance liquid chromatographic system (HPLC), consisting of a Waters Model 600-A solvent delivery system, a Rheodyne 7125 injector, a Waters μBondapak C_{18} column (3.9-mm i.d. × 300 mm), and a Spectra-Physics SP 8450 UV/vis detector operated at 360 nm. The mobile phase consisted of acetonitrile, tetrahydrofuran, and water (60:5:35) and the retention time was 3.0 min at 1.5 ml/min flow rate. All determinations were made at ambient temper-

Table I. Experimental Conditions and Pseudo-First-Order Rate Constants (k_{obs}) for Overall Loss of 1[a]

pH ± SD	Buffer (M)[b]		k_{obs} (min^{-1})[c]	No. of experiments
	Acetic acid	Sodium acetate		
4.57 ± 0.04	0.400	0.400	0.2978 ± 0.0210	4
	0.300	0.300	0.2452 ± 0.0130	4
	0.200	0.200	0.1853 ± 0.0098	4
	0.100	0.100	0.1477 ± 0.0085	4
4.70 ± 0.4	0.300	0.400	0.2824 ± 0.0206	3
	0.225	0.300	0.2300 ± 0.0181	3
	0.150	0.200	0.1852 ± 0.0103	3
	0.075	0.100	0.1227 ± 0.0032	3
5.68 ± 0.05	0.030	0.300	0.0400 ± 0.0011	3
	0.020	0.200	0.0310 ± 0.0001	3
	0.010	0.100	0.0198 ± 0.0007	3
	Sodium phosphate monobasic	Sodium phosphate dibasic		
5.75 ± 0.04	0.058	0.012	0.0206 ± 0.0015	3
	0.044	0.009	0.0169 ± 0.0001	3
	0.029	0.006	0.0130 ± 0.0012	3
6.50 ± 0.05	0.058	0.058	0.0165 ± 0.0008	3
	0.048	0.048	0.0139 ± 0.0018	3
	0.029	0.029	0.0088 ± 0.0004	3
6.93 ± 0.04	0.037	0.089	0.0133 ± 0.0002	3
	0.0185	0.0445		
0.0072 ± 0.0003	0.0092	0.022	0.0053 ± 0.0002	3
	Boric acid	Sodium borate		
8.07 ± 0.03	0.100	0.0125	0.0289 ± 0.0008	4
	0.0693	0.00867	0.0305 ± 0.0002	4
	0.0500	0.00625	0.0308 ± 0.0013	4
8.74 ± 0.02	0.038	0.056	0.1340 ± 0.0080	3
	0.025	0.038	0.1351 ± 0.0070	3
	0.012	0.019	0.1124 ± 0.0101	3
8.77 ± 0.03	0.075	0.075	0.1179 ± 0.0070	3
	0.050	0.050	0.1113 ± 0.0060	3
	0.025	0.025	0.1133 ± 0.0060	3

[a] Temperature, 40.0°C; ionic strength, 0.5 M; initial concentration of 1, 1×10^{-4} M.
[b] Ionic strength, 0.5 (with NaCl).
[c] Mean ± SD values.

Table II. The Effect of Ionic Strength (μ) on the Pseudo-First-Order Rate Constant (k_{obs}) for Overall Loss of 1 from a 0.38 *M* Aqueous Phosphate Buffer Solution Containing 0 and 1% 2-Hydroxypropyl-β-cyclodextrin (HPCD)[a]

	k_{obs} (min^{-1})	
μ	0% HPCD	1% HPCD
0.10	0.0066	0.0020
0.20	0.0067	0.0022
0.30	0.0066	0.0020
0.40	0.0072	0.0019
0.50	0.0073	0.0021

[a] Temperature, 40.0°C; pH 6.62; ionic strength adjusted with sodium chloride.

Table III. Influence of Oxygen-Free Reaction Conditions on the Pseudo-First-Order Rate Constant (k_{obs}) for the Overall Loss of 1 from a 0.02 *M* Aqueous Phosphate Buffer Solution Containing Various Amounts of 2-Hydroxypropyl-β-cyclodextrin (HPCD)[a]

	k_{obs} (min^{-1})	
HPCD	Normal condition	Nitrogen-saturated medium
0%	0.0072	0.0073
0.5%	0.0022	0.0021
1%	0.0017	0.0015
2%	0.0014	0.0013
5%	0.0011	0.0012

[a] Temperature, 40.0°C; pH 6.82; ionic strength, 0.10.

ature. Standard curves for 1 were linear ($r > 0.999$) over the examined concentration ranges.

Kinetic Studies; Stability of 1–HPCD Solutions

Kinetic studies were carried out by adding a stock solution of 1 in acetonitrile to 3.0 ml of appropriate reaction medium, previously equilibrated at the desired temperature for at least 30 min. The initial concentration of 1 was 1×10^{-4} *M*. The acetonitrile concentration in the final solution was 0.7%; the HPCD concentrations ranged from 0 to 5% (w/v). The experimental conditions were as described in Tables I–IV. Unless otherwise indicated, the ionic strength (μ) was maintained at 0.5 using sodium chloride. The pH values were measured at the reaction temperature. All reactions were run under pseudo-first-order conditions. Aliquots (50 μl) were taken at various times after sample preparation and injected (20 μl) directly into the HPLC column. Rate constants were determined from the disappearance of compound by linear regression of natural logarithm of the peak height-versus-time plots. Several representative pseudo-first-order plots for the degradation of 1 are illustrated in Fig. 1.

RESULTS AND DISCUSSION

The benzylpenicillin chemical delivery system 1 was obtained with improved yields and quality as compared to the original procedure (20) by using a modified method. The intermediate benzylpenicillin nicotinate resulted with 80% yield by using a one-step method (reaction of benzylpenicillin with chloromethylnicotinate), as compared to 50% obtained by the originally described two-step procedure (benzylpenicillin → chloromethylbenzylpenicillin → nicotinate). The global yield of 1 was improved in this way from 32 to 50%. The ability of HPCD to enhance the aqueous solubility of 1 was examined. Due to the relative instability of 1 in water, a reduced sonication time was employed as compared to the generally applied methodology (28); the optimum sonication and equilibrium times, i.e., the conditions under which minimum degradation of 1 occurred, were determined in a preliminary experiment. The penicillin derivative was then added to aqueous solutions of HPCD of various concentrations and sonicated for 30 min, and after another 30 min of equilibration at 30°C, the concentration of 1 in the solution was determined by HPLC. Each experiment was repeated several times and the reported results represent average values of at least two determinations. The solubility of 1 in water in the absence of HPCD is approximately 50–70 ng/ml (the instability of 1 could not allow for a more accurate determination).

Figure 2 represents the phase solubility of 1 in aqueous HPCD. As expected, the solubility of 1 increased as a linear function of the HPCD concentration. The solubility curve can be classified as type A_L, i.e., the complex formed has a

Table IV. Effect of HPCD Concentration on the Pseudo First-Order Rate Constant (k_{obs}) for the Overall Loss of 1[a]

Buffer		pH ± SD	k_{obs} (min^{-1}) for HPCD % (w/v)			
			0	0.5	1	5
Acetic acid	Sodium acetate					
0.100	0.100	4.66 ± 0.01	0.1448	0.0460	0.0329	0.0191
Sodium phosphate monobasic	Sodium phosphate dibasic					
0.019	0.019	6.87 ± 0.01	0.0072	0.0030	0.0022	0.0016
Boric acid	Sodium borate					
0.050	0.00625	8.36 ± 0.01	0.0418	0.0247	0.0215	0.0233
0.0025	0.025	8.91 ± 0.01	0.1477	0.0903	0.0830	0.0760

[a] Temperature of determination, 40.0°C; ionic strength was not adjusted.

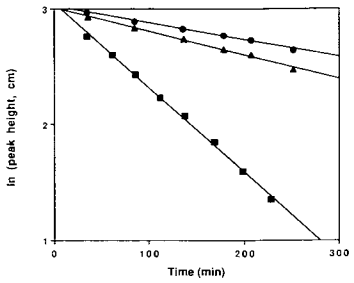


Fig. 1. Pseudo-first-order plots for the degradation of 1 in pH 6.93 aqueous phosphate buffer (0.37 *M*), ionic strength 0.5, at 400°C and concentrations of HPCD as follows (w/v): 0.0% (■); 0.5% (▲); 1.0% (●).

first-order dependence on the HPCD concentration. The concentrations of HPCD used in this study were in the range of 2–20% (w/v) and the solubility of 1 increased dramatically, reaching 4.2 mg/ml at a concentration of 20% HPCD (meaning about 20 mg of 1 per g of solid dry 1–HPCD complex). However, the solubilization can be enhanced even more by using cosolvents (such as ethanol) during the solubilization process. Incorporations of about 30 mg 1 per 1 g dry complex could be achieved in this way. Filtered aqueous solutions of the complex could be conveniently lyophilized to yield the dry powder-like solid complexes. The solid material can facilitate preparation of formulations.

The stability of 1 and the effects of HPCD on it were studied. The degradation of 1 in aqueous solution can follow one of the pathways indicated in Scheme II: oxidation, hydrolysis, which occurs mainly at basic pH's, and acid-catalyzed hydration (20). Only these main degradation processes were considered here, since other possible transformations such as degradations of the β lactamic system or of the dihydrothiazine ring moiety, which generally occur under the influence of alkalis or strong mineral acids, are insignificant under the relatively mild conditions used during this investigation. The oxidation of 1 to the quaternary salt form was previously examined (20). A ferricyanide-mediated oxidation study indicated that 1 was rather stable toward oxidation, having a second-order oxidation rate constant of 0.530 M^{-1} sec^{-1}. However, the influence of oxygen-free conditions on the degradation process was investigated and is discussed later. Base-catalyzed hydrolysis of the gem-diol diesters or acyloxyalkyl esters such as 1 (or 2 and 5) should be a two-step process (29); the first, rate-determining, step is the hydrolysis of the 1-methylpyridinium-3-carboxylate ester linkage with formation of the unstable hydroxymethyl ester of the benzylpenicillin 3, which subsequently decomposes spontaneously into benzylpenicillin (4) and formaldehyde. Water addition to the dihydropyridine nucleus in the presence of acids involves protonation of C-5 as a rate-determining step, followed by rapid nucleophilic attack of OH^- at C-6, resulting in the 6-hydroxy-1,4,5,6-tetrahydropyridine derivative 5. The degradation products were identified by UV spectroscopy and HPLC.

The pH profile for the overall loss of 1 at ionic strength of 0.5 *M*, zero buffer concentration, and 40°C follows pseudo-first-orderkinetics and is indicated in Fig. 3. Each experiment was repeated several times and the values of k_{obs} reported in Table I are the average ones. These values were used to calculate the observed rate constants at zero buffer concentrations (k^1 obs), which were used to construct the profile in Fig. 3. As it was previously proven, at acidic pH (20,21,32,33), the hydration of the dihydropyridine 5,6 double bond is the main degradation pathway, the resultant water addition product, the 6-hydroxy-1,4,5,6-tetrahydropyridine derivative, being identified by its characteristic UV absorption wavelength at 290 nm, which replaces the 360-nm absorption maxima typical for the 1,4-dihydropyridine derivative 1. At basic pH mainly hydrolysis of 1 occurs. The maximum stability of 1 is at pH 6.5–7.

Changes in the ionic strength (Table II) did not affect the reaction rate. By performing the determination under oxygen-free conditions (by saturating the reaction medium with nitrogen), the overall loss was practically unchanged (Table III), which is explained by the already mentioned slow oxidation rate of 1 to the quaternary salt 2. Evidently, oxidation is not an important degradation pathway of 1.

The influence of HPCD on the stability of 1 was determined by increasing the concentrations of HPCD (1–5%) in the reaction medium at various pH's (4.5–9) and determining the first-order rate constants for the overall loss of 1 (k_{obs}). The results are presented in Table IV. A nonlinear relationship between k_{obs} and HPCD concentrations was obtained. The rate decreased fast when the HPCD concentration was increased from 0 to 0.5%, then it leveled off at higher HPCD concentrations. The results are in agreement with kinetic systems where a compound degrades at a higher rate outside than inside the complex. The complexation equilibrium and outside-the-complex and inside-the-complex degradations are illustrated by the following scheme.

$$\begin{array}{ccc} & K_c & \\ 1 + \text{HPCD} & \longleftrightarrow & \text{1–HPCD} \\ \downarrow k_o & & \downarrow k_c \\ \text{Degradation products} & & \end{array}$$

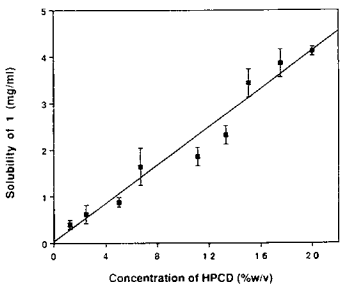


Fig. 2. Phase-solubility diagram of 1 in aqueous HPCD solution at 30°C.

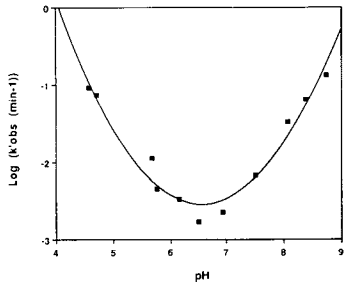


Fig. 3. The pH–rate profile for overall loss of 1 at ionic strength of 0.5 *M*, zero buffer concentration, and 40°C.

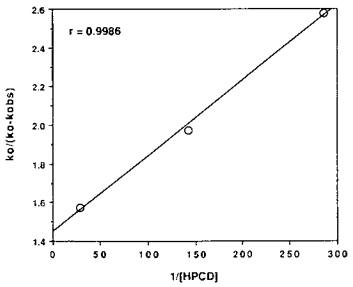


Fig. 4. A representative Lineweaver–Burk plot for 1 in aqueous HPCD, 0.06 *M* phosphate buffer solution at pH 6.45 and ionic strength 0.5 (NaCl).

where k_o represents the pseudo-first-order rate constant for the degradation of the free compound (when no HPCD is present), k_c the pseudo-first-order rate constant for the degradation of the drug in complex, and K_c the stability constant of the inclusion complex, assuming 1:1 complexation. Knowing k_o, k_c, and K_c can be calculated after construction of Lineweaver–Burk plots using the equation (29,30)

$$\frac{k_o}{k_o - k_{obs}} = \frac{k_o}{K_c(k_o - k_c)[HPCD]} + \frac{k_o}{(k_o - k_c)}$$

k_c being obtained from the ordinate intercept and K_c by dividing the slope into the ordinate intercept. The correlation coefficient of the linear plots obtained were in all cases greater than 0.998. A representative plot is shown in Fig. 4.

The values of k_c and K_c are indicated in Table V. It is obvious that HPCD stabilized the chemical delivery system, especially at acidic and neutral pH (9- to 11-fold at acidic and about 6-fold at neutral). This is of importance, particularly if oral administration of 1 is considered. The stability constant (K_c) of the 1–HPCD complex was in the range 720 to 940 M^{-1}.

The effect of temperature on the rate of degradation of 1 was investigated. The activation parameters for the degradation of 1, both within the complex and out in the solution, and the enthalpy for the complex formation are displayed in Table VI. The enthalpy change for the complex formation is negative, resulting in a decrease in the free energy through the complexation process. The activation parameters for the degradation result, on the other hand, in an increase in the free energy. Thus, when the temperature of the HPCD-containing reaction medium is lowered, both the decrease in the degradation rate constant and the increase in the stability constant for the inclusion complex will result in stabilization of 1.

Table V. Pseudo-First-Order Rate Constants for the Overall Loss of Free 1 (k_o) and Complexed 1 (k_c) and the Stability Constant of the Complex (K_c)[a,b]

pH ± SD	k_o (min^{-1})	k_c (min^{-1})	k_o/k_cK_c (M^{-1})	
4.66 ± 0.01	0.1448	0.0127	11.4	936.75
6.87 ± 0.01	0.0072	0.0013	5.5	721.71
8.36 ± 0.01	0.0418	0.0182	2.3	768.70
8.91 ± 0.01	0.1477	0.0696	2.1	788.33

[a] Temperature, 40.0°C; ionic strength, not adjusted.
[b] A 1:1 complexation is assumed.

Table VI. The First-Order Rate Constants, the Stability Constants, and the Activation Parameters for the Degradation of 1[a]

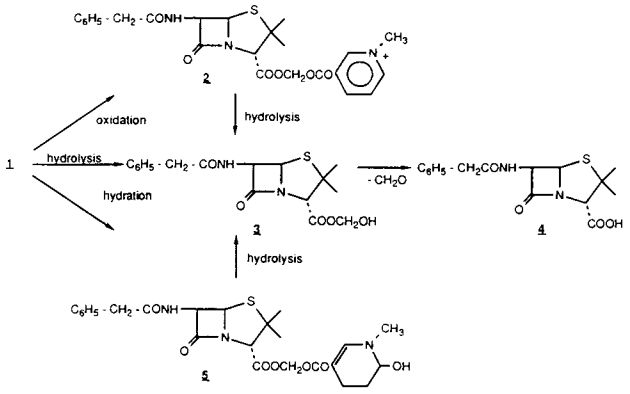
	$k_o \times 10^2$ (min^{-1})	$k_c \times 10^2$ (min^{-1})	K_c (M^{-1})
Temperature (°C)			
70.0	10.42	4.45	811.0
62.1	5.31	1.86	303.9
51.5	2.11	0.73	181.9
$\Delta H \neq$ (kJ/mol)[b]	77.1	87.0	−50.2
$\Delta S \neq$ (J/mol/deg)[c]	−6.3	−15.1	

[a] Aqueous, buffered HPCD solution at pH 6.45 ± 0.05 (at 0.06 *M*, ionic strength 0.5 *M*).
[b] Heat of activation.
[c] Entropy of activation.

In summary, aqueous solutions of HPCD solubilized the benzylpenicillin chemical delivery system 1 in amounts which could be useful pharmacologically. The increased stability of the complexed 1 and the low toxicity of the HPCD (34) may lead to a practical formulation modality which could be an alternative to already-studied vehicles (21,22) for parenteral administration of this compound.

NOMENCLATURE

HPCD	2-Hydroxypropyl-β-cyclodextrin
CNS	Central nervous system
BBB	Blood–brain barrier
1–HPCD	Inclusion complex of the benzylpenicillin chemical delivery system 1 and 2-hydroxypropyl-β-cyclodextrin
HPLC	High-performance liquid chromatography
k_{obs}	Pseudo-first-order rate constant for the overall loss of 1
k_o	Pseudo-first-order rate constant for the overall loss of free 1 (when no HPCD is present)
k_c	Pseudo-first-order rate constant for the overall loss of complexed 1
K_c	Stability constant of the 1-HPCD complex



Scheme II

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