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Synthesis of New Covalently Bound κ-Carrageenan−AZT Conjugates with Improved Anti-HIV Activities

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This paper describes the first covalent synthesis of κ-carrageenan−3′-azido-3′-deoxythymidine (AZT) conjugates. A succinate diester spacer was used to covalently couple AZT onto κ-carrageenan, resulting in a tripartite prodrug. Two methods (UV and radioactive counting) are described and validated to determine the AZT loading onto the κ-carrageenan carrier. This polymeric carrier, through its own intrinsic anti-HIV activity, is expected to act not only as a drug delivery agent but also as an anti-HIV agent. Synergism between the two drugs (κ-carrageenan and AZT) was demonstrated when MT-4 cells were preincubated with the κ-carrageenan−AZT conjugate prior to HIV-1-infection. A threshold of AZT loaded onto the κ-carrageenan was required to achieve this synergistic effect. Such κ-carrageenan−AZT conjugates could be of great therapeutic interest because these conjugates, which contain a low AZT concentration, present improved anti-HIV activities relative to free AZT. Moreover, κ-carrageenan is a well-tolerated biopolymer, already used in the food industry.

Introduction

Intensive efforts are underway worldwide to develop chemotherapeutic agents effective against the human immunodeficiency virus (HIV),[1,2,3] the etiological agent of the acquired immunodeficiency syndrome (AIDS).[4] The search for an effective chemotherapeutic treatment against HIV infection has led to the development of agents that target specific and critical events in the HIV replicative cycle.[5,6] Among the current diversity of compounds active against HIV, the 2′,3′-dideoxynucleosides (ddNs) remain by far the most potent.[7−9] The most extensively studied of these agents is 3′-azido-3′-deoxythymidine (AZT, zidovudine, Retrovir; Figure 1).[10] This ddN was the first that was approved by the Food and Drug Administration[11] for the treatment of AIDS.[12,13] AZT, after conversion into its 5′-*O*-triphosphate analogue (AZT-TP) by cellular enzymes (kinases),[14−16] inhibits HIV reverse transcriptase (HIV-RT) by competitive inhibition of the viral reverse transcriptase (RT) and/or incorporation and subsequent chain termination of the growing viral DNA strand.[17] The major limitations of AZT are due to clinical toxicities[18−23] and to the development of AZT resistance by HIV.[9,24−31] In attempts to overcome these problems, numerous chemical strategies have been developed by medicinal chemists for designing AZT prodrugs (generally 5′-*O*-carboxylic esters).[10] The mechanism of action of these ester conjugates is based on hydrolysis and/or enzymatic cleavage of their 5′-O-bonds, between the drug (AZT) and its spacer group, to AZT within the cells. The expected advantages of these 5′-O-substitued AZT prodrugs can be multiple: improvement in anti-HIV activity,[10] synergistic drug interactions,[32−34] enhancement of AZT intracellular uptake,[35−37] and decrease of toxicity.[10]

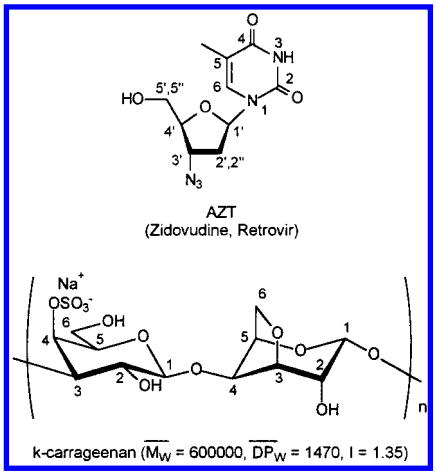

Figure 1. Structures of AZT, the first FDA-approved drug for HIV-treatment, and κ-carrageenan, one of the first sulfated polysaccharides found to be active against HIV in vitro.

Moreover, free AZT is known to act synergistically with numerous compounds, including sulfated polysaccharides.[38−43] Taking into account these findings, we considered a strategy to synthesize new covalent conjugates between κ-carrageenan and AZT.

Carrageenans are natural sulfated polysaccharides extracted from different species of red seaweed that have a common structural backbone of D-galactose residues. The three families of carrageenans are the λ family (λ-, π-, and ϵ-), the β family (β- and γ-), and the κ family (μ-, ν-, κ-, and ι-). The κ-form is characterized by a repeating unit of 4-sulfate-β-D-galactopyranose linked 1→3 and 3,6-anhydro-α-D-galactopyranose linked

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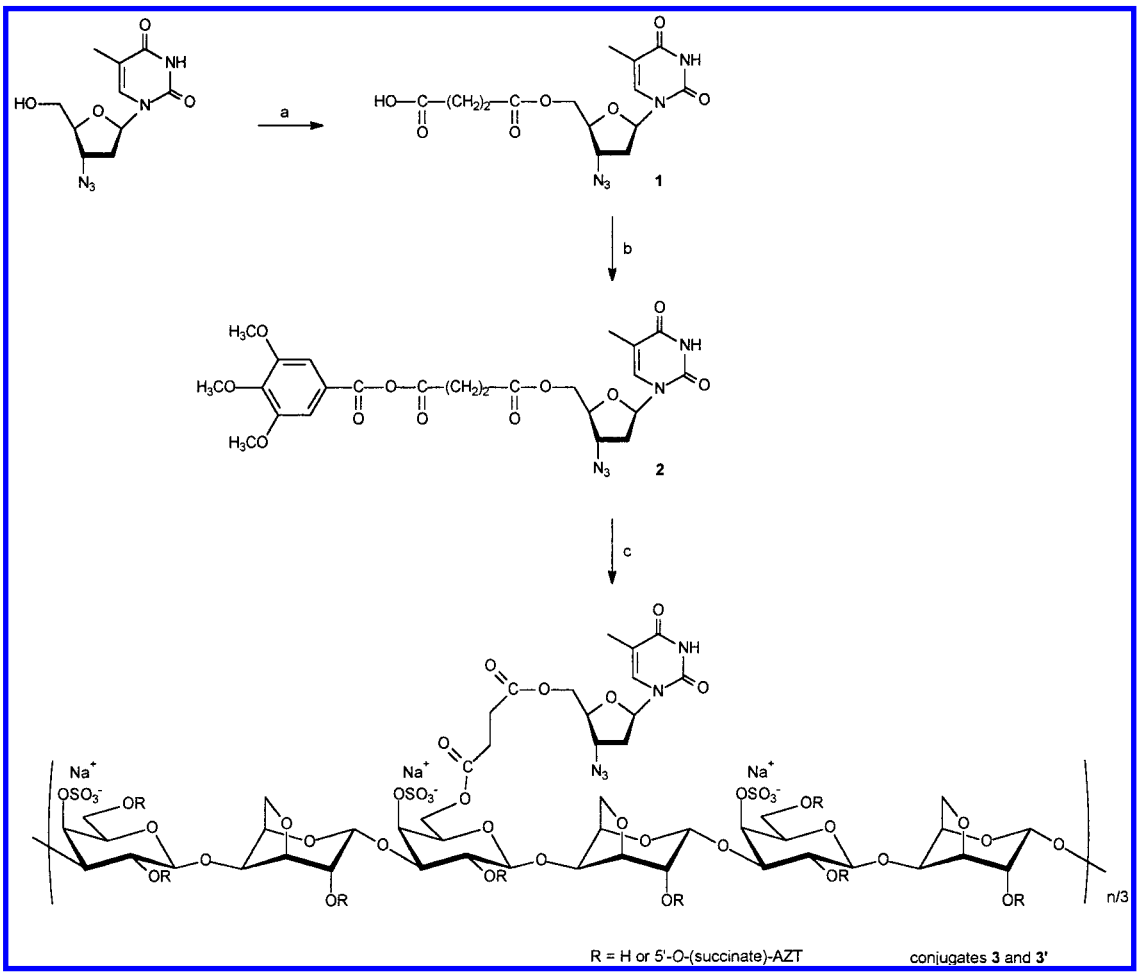
Scheme 1[a]



[a] Reagents: (a) succinic anhydride, DMAP, DMF; (b) 3,4,5-trimethoxybenzoyl chloride, Et_3N, EtOAc; (c) κ-carrageenan, DMF, 60 °C, 72 h.

1→4. The degree of sulfation for the κ-form is 25% (Figure 1).[44]

An ideal drug carrier (ideally a biomacromolecule) must (i) protect the drug until it is delivered to the site of action, (ii) localize the drug at or near the site of action, (iii) allow the drug release through a chemical and/or an enzymatic pathway, (iv) minimize host toxicity, (v) be biodegradable, biochemically inert, and nonimmunogenic, (vi) be easily prepared inexpensively, and (vii) be chemically and biochemically stable in its formulation.[45]

In our concept, κ-carrageenan is expected to act not only as a drug delivery carrier (for AZT release within or near the infected cells) but also as an anti-HIV agent by itself, which could act synergistically with AZT. Indeed, carrageenans such as other sulfated polysaccharides were found to be active against a wide variety of enveloped virus.[44,46] Carrageenans were among the first sulfated polysaccharides found to possess anti-HIV activity.[47−49] They inhibit the binding of the virions to the cell (by shielding the positively charged sites of amino acids in the V3 loop of the viral envelope glycoprotein (gp120))[46,50] and the cell-to-cell fusion.[51] Moreover, carrageenans seem to possess a combination of good anti-HIV-1 activities and low anticoagulant properties (in contrast with heparin) that may justify clinical trials for their therapeutic potential (oral absorption).[44,52−54]

The combination of different antiviral drugs (e.g., mutual tripartite prodrugs)[45] that target different events in the HIV replicative cycle (e.g., virus attachment and RT) has a triple aim: (i) it may lead to increased activity (ideally synergism); (ii) it may allow reduction of the individual doses and thus decrease drug toxicity; (iii) it may prevent or delay the emergence of drug-resistant virus strains.[46]

This paper describes, to our knowledge, the first synthesis of κ-carrageenan−AZT conjugates,[55] the methods used to determine the AZT loading onto the carrier, and their in vitro anti-HIV activities correlating with some of their biophysical properties.

Chemistry

We describe herein the synthesis of κ-carrageenan−AZT conjugates via a succinate diester linkage (Scheme 1).

Prior to the coupling of AZT onto the κ-carrageenan moiety, 5′-*O*-(succinate)-AZT **1** was first synthesized by acylation of the 5′-hydroxyl group of AZT with succinic anhydride in DMF in the presence of 4-(dimethylamino)-pyridine (DMAP) in 85% yield.[37]

Since carrageenans are highly soluble in aqueous solutions, we first tried to couple **1** onto the κ-carrageenan carrier in water using *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) as an

activating agent, which is well-known to activate carboxylic acids in aqueous medium. Unfortunately, the AZT loading onto the κ-carrageenan could not be determined by a UV spectrophotometric technique (measuring absorption at 266 nm) because of the strong absorption (interference) of EDCI probably complexed with the negatively charged sulfate groups of κ-carrageenan. The same problems of spectroscopic interference were encountered when we attempted to link covalently **1** onto the κ-carrageenan in DMF (in which carrageenans swell but do not dissolve, like some polymers used in solid-phase synthesis) using various activating agents: dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt), DCC/pentachlorophenol (Pcp)/HOBt, DCC/*N*-hydroxysuccinimide (HOSu), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphonate (BOP), or 1,1′-carbonyldiimidazole (CDI). Unfortunately, each assay led to abnormal UV absorbancies (interference), which impeded the determination of the amount of AZT covalently bound to the κ-carrageenan carrier. Finally, we utilized a chemical strategy involving the formation of a mixed anhydride using the 3,4,5-trimethoxybenzoyl group as the leaving part of the anhydride without another activating agent.[56] This kind of strategy has never been described for the coupling of drugs onto a carrageenan carrier.

In fact, conjugation of carboxylic acid **1** onto κ-carrageenan was achieved through the formation of a mixed anhydride **2** using 3,4,5-trimethoxybenzoyl chloride in the presence of triethylamine (Et_3N) in EtOAc in 94% yield (Scheme 1).[56] The resulting anhydride **2** was conjugated onto the κ-carrageenan in DMF at 60 °C over 72 h to give the κ-carrageenan−AZT conjugates **3** and **3′** via a succinate diester linkage (Scheme 1). Conjugates **3** and **3′** only differed by the percentage of AZT coupled onto the κ-carrageenan. These new coupling conditions make it possible to determine the AZT loading onto the κ-carrageenan through a simple UV spectrophotometric technique (at 266 nm) without any interference due to the remaining coupling reagent, to byproducts of the reaction (removed by filtration after the precipitation of the covalent conjugate), or to κ-carrageenan. Then, we investigated what was the optimum ratio of the mixed anhydride **2** to the κ-carrageenan (equivalent amount of mixed anhydride **2** versus equivalent amount of κ-carrageenan calculated on its repeating disaccharidic unit) to obtain the best procedure for AZT loading onto the κ-carrageenan moiety.[57] We found that conjugate **3** synthesized using a ratio of 1.25 equiv of AZT to 1 equiv of κ-carrageenan led to an AZT loading of $2.0 \pm 0.5 \times 10^{-5}$ mmol of AZT covalently bound to 1 mg of κ-carrageenan. However, the best procedure for AZT loading onto the κ-carrageenan (conjugate **3′**) was obtained using a ratio of 10 to 1, respectively. This synthesis led to an AZT loading of $(6.8 \pm 0.5) \times 10^{-5}$ mmol of AZT covalently bound to 1 mg of κ-carrageenan (as described below).

Blanks consisting of mixtures of 5′-*O*-(succinate)-AZT and κ-carrageenan (treated under the same conditions used for the corresponding covalent coupling reactions but in absence of activating agents) were always performed for UV determination of the AZT covalent loading onto the κ-carrageenan. Moreover, as expected, no free AZT derivatives were detected from the UV reading of the blank assays.

To confirm the AZT loading onto the κ-carrageenan moiety, the synthesis of the corresponding κ-carrageenan−[2-^{14}C]-AZT conjugate **3′** was achieved. For this synthesis, a total of 808 nmol of [2-^{14}C]-AZT was added to 4.5 mmol of AZT (50 mCi/mmol), which was coupled onto the κ-carrageenan via the synthesis of the 5′-*O*-(succinate)-[2-^{14}C]-AZT precursor.

Results and Discussion

The succinate diester linkage for conjugation between κ-carrageenan and AZT was initially selected with the thought that release of AZT would be facilitated through the action of esterases in human serum (normal human serum, NHS). Indeed, from literature data, it has already been demonstrated that tripartite prodrugs containing the succinate diester linkage are hydrolyzed more rapidly than their corresponding succinate monoesters (Figure 2).[45] The determination of the AZT loading onto the κ-carrageenan carrier was required in order to study the sensitivity to enzymatic hydrolysis and the anti-HIV activities of conjugates **3** and **3′**. Two different techniques (UV and radioactive counting) were used for the quantification of the AZT loading onto the κ-carrageenan carrier.

AZT Loading onto the κ-Carrageenan. (i) UV Determination. AZT content in the κ-carrageenan−succinate diester−AZT conjugates **3** and **3′** was determined by measuring the absorption at 266 nm, which is the absorption maximum for AZT. κ-Carrageenan does not absorb at this wavelength. We first established a standard curve at 266 nm, plotting absorbancies (OD) versus variable AZT concentrations in water in the presence of a constant amount of κ-carrageenan (1 mg/mL). This standard curve was linear ($r = 1.000$) with respect to AZT concentration up to 90 μM (data not shown). The AZT loading on each conjugate sample was determined by interpolation of the standard curve at 266 nm with a conjugate concentration of 1 mg/mL.

This method (performed in triplicate assays) allowed us to determine that conjugates **3** and **3′** contained $(2.0 \pm 0.5) \times 10^{-5}$ and $(6.8 \pm 0.5) \times 10^{-5}$ mmol of AZT covalently bound to 1 mg of κ-carrageenan, respectively. As reported previously for curdlan sulfate,[58] the esterification occurred predominately at the primary hydroxyl function in this polysaccharidic family (hydroxyl function in the 6-C position of the 4-sulfate-β-D-galactopyranose part of the κ-carrageenan disaccharidic unit). In fact, a repeating disaccharidic unit of κ-carrageenan possesses only one primary hydroxyl function likely to be esterified. So the number of primary hydroxyl functions per milligram of κ-carrageenan is equal to the number of mmoles represented by 1 mg of κ-carrageenan (2.45×10^{-3}). For conjugates **3** and **3′**, the degree of AZT substitution calculated on a repeating disaccharidic unit is only 0.8% and 2.8%, respectively.

From these results, it can be deduced that an 8-fold excess of anhydride **2** results in a three-and-one-half-fold increase of the AZT loading onto the κ-carrageenan carrier (conjugate **3′** versus conjugate **3**). This apparent weak AZT loading onto the κ-carrageenan carrier could be explained in part by the gelling properties of κ-carrageenan, which forms double helices in solution, and

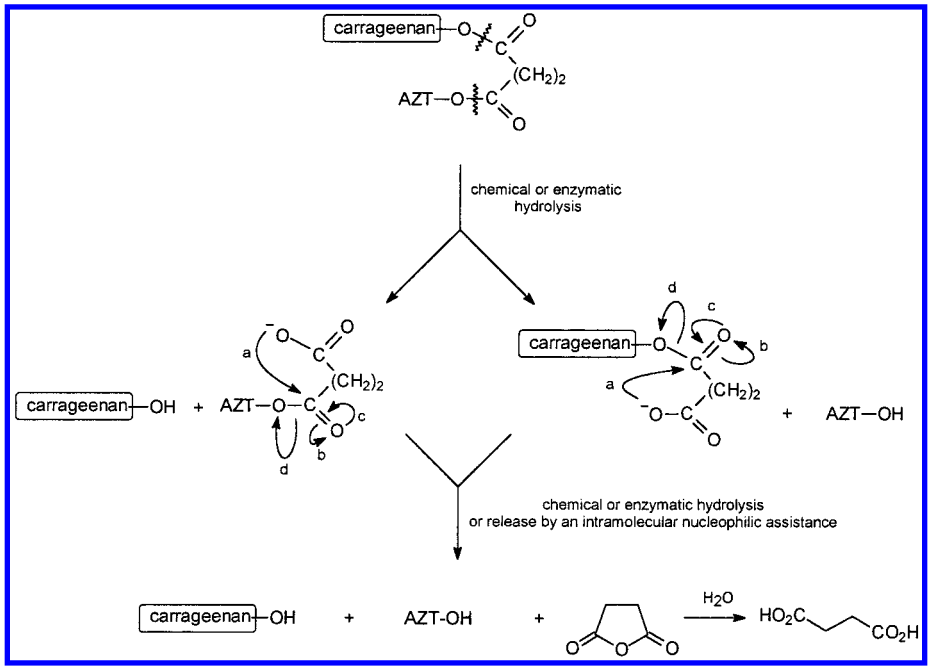


Figure 2. Concept of tripartite prodrugs applied to AZT and κ-carrageenan.

Table 1. Chemical and Enzymatic Stability of Prodrug **1** and Conjugate **3′** in Acid, Neutral, and Basic Buffers and in Human Serum (NHS)

	% of nucleoside derivatives released (AZT in the case of compound **1**; AZT and compound **1** in the case of conjugate **3′**) after 48 h of hydrolysis					
compd	citric acid, pH 1.5, 0.1 M	acetate buffer, pH 5.5, 0.1 M	PBS buffer, pH 7.4, 0.1 M	PBS buffer, pH 8.5, 0.1 M	NaOH, pH 13.0, 0.5 M	human serum (NHS), pH 7.2
1	16 ± 0.5	4 ± 0.5	5 ± 0.5	21 ± 0.5	100	100[a]
3′	25 ± 0.5	6 ± 0.5	8 ± 0.5	33 ± 0.5	100	39 ± 0.5

[a] $t_{1/2}$ (half-life), the time required for 50% hydrolysis of prodrug **1** to AZT at 37 °C upon incubation in NHS, was 25 h.

by the steric hindrance between the two polymeric units of the helix.[59,60]

(ii) Radioactive Counting Determination. To confirm the above results, we performed the radiolabeled chemical synthesis of conjugate **3′** with [2-^{14}C]-AZT using the same conditions as previously described. The radioactive counting (triplicate assays) indicated that (5.7 ± 0.5) × 10^{-5} mmol of AZT was covalently bound to 1 mg of κ-carrageenan. Moreover, UV determination (triplicate assays) of this radiolabeled conjugate showed that (5.9 ± 0.5) × 10^{-5} mmol of AZT was covalently bound to 1 mg of κ-carrageenan. In conclusion, radiolabeled chemical synthesis of conjugate **3′** with [2-^{14}C]-AZT confirmed the results obtained by UV spectrophotometry, in agreement with our previous findings.

Stability Studies in Acid and Basic Buffers and in Human Serum (NHS).[61,62] In Table 1, the stabilities of prodrug **1** and conjugate **3′**, incubated for 48 h at 37 °C, in acid and basic buffered solutions and in human serum are summarized. The results obtained indicate the following observations. (i) Under strong acid conditions, at pH 1.5 (citric acid, 0.1 M), simulating gastric juice, conjugate **3′** was slightly more sensitive to hydrolysis (25% of hydrolysis products released: AZT and compound **1**) than prodrug **1** (16% of AZT released) (Table 1). (ii) Under weakly acid conditions, at pH 5.5 (acetate buffer, 0.1 M), mimicking endosomal compartments, prodrug **1** and conjugate **3′** appeared to be quite stable. HPLC analytic profiles showed that for prodrug **1**, release of AZT was less than 4% and that for conjugate **3′**, for which two hydrolysis products can be expected (AZT and compound **1**), the total amount of hydrolysis products was less than 6%. (iii) Under neutral conditions (PBS buffer, pH 7.0, 0.1 M), prodrug **1** and conjugate **3′** appeared to be very stable. No residual-free AZT or prodrug **1** was detected by HPLC after conjugate **3′** was incubated over 48 h under these conditions (data not shown). (iv) In weakly basic conditions, at pH 7.4 (PBS buffer, 0.1 M), simulating extracellular fluids, prodrug **1** and conjugate **3′** appeared to be quite stable. HPLC analytic profiles showed that for prodrug **1**, release of AZT was less than 5% and that for conjugate **3′**, for which two hydrolysis products can be expected (AZT and compound **1**), the total amount of hydrolysis products was less than 8%. The whole results clearly indicate that the ester bonds between AZT and κ-carrageenan were stable in neutral and weakly acid or basic aqueous solutions (Table 1). (v) In contrast, under strong basic conditions (pH 13.0), both compounds (prodrug **1** and conjugate **3′**) were totally hydrolyzed in a base-dependent manner to give 100% of free AZT. This alkaline hydrolysis permits us to confirm by HPLC analysis the total amount of AZT linked onto κ-carrageenan previously determined by UV and radioactivity. At pH 8.5 (PBS buffer, 0.1 M), conjugate **3′** was slightly more sensitive to hydrolysis (33% of hydrolysis products released) than prodrug **1** (21% of AZT released) (Table 1). These results are in accordance with the concept of tripartite prodrugs via

Table 2. Anti-HIV Evaluation of Prodrug **1** and Conjugates **3** and **3′** in MT-4 Cells[a] Infected with HIV-1 (Strain BRU)

compd	EC_{50}[b] (μg/mL)	CC_{50}[c] (μg/mL)	mmol of AZT/ mg of κ-carrageenan[d]	EC_{50}[e] (nM)	CC_{50}[f] (μM)	SI[g]	FIC[h]
1				15	>50	>3333	
3	4	300	2.0×10^{-5}	80	*i*	75	3.60
3′	0.1	300	6.8×10^{-5}	6.8	*i*	3000	0.28
κ-carrageenan	10	300				30	
AZT				25	>100	>4000	

[a] MT-4 cells were pretreated with antiviral compounds before HIV-1-infection (see Anti-HIV Activity Assays, (i), in Experimental Section). [b] EC_{50}: concentration in μg/mL (expressed in κ-carrageenan equivalent) required to inhibit the cytopathicity of HIV-1 by 50% in MT-4 cells. [c] CC_{50}: concentration in μg/mL (expressed in κ-carrageenan equivalent) required to cause 50% death of uninfected MT-4 cells. [d] Amount of AZT covalently bound onto κ-carrageenan. [e] EC_{50}: concentration in nM (expressed in AZT equivalent) required to inhibit the cytopathicity of HIV-1 by 50% in MT-4 cells. [f] CC_{50}: concentration in μM (expressed in AZT equivalent) required to cause 50% death of uninfected MT-4 cells. [g] SI: selectivity index = CC_{50}/EC_{50}. [h] FIC: fractional inhibitory concentration. [i] Not calculable because the cytotoxicity of these conjugates are only due to their κ-carrageenan moieties at the concentration of 300 μg/mL.

Table 3. Anti-HIV Evaluation of Prodrug **1** and Conjugate **3′** in MT-4 Cells[a] Infected with HIV-1 (Strain III_B)

compd	EC_{50}[b] (μg/mL)	CC_{50}[c] (μg/mL)	mmol of AZT/ mg of κ-carrageenan[d]	EC_{50}[e] (nM)	CC_{50}[f] (μM)	SI[g]	FIC[h]
1				65	130	2000	
3′	1.6	300	6.8×10^{-5}	109	*i*	188	9.22
κ-carrageenan	12	300				25	
AZT				12	42	3500	

[a] MT-4 cells were infected by HIV-1 and then treated with antiviral compounds (see Anti-HIV Activity Assays, (ii), in Experimental Section).[70] [b] EC_{50}: concentration in μg/mL (expressed in κ-carrageenan equivalent) required to inhibit the cytopathicity of HIV-1 by 50% in MT-4 cells. [c] CC_{50}: concentration in μg/mL (expressed in κ-carrageenan equivalent) required to cause 50% death of uninfected MT-4 cells. [d] Amount of AZT covalently bound onto κ-carrageenan. [e] EC_{50}: concentration in nM (expressed in AZT equivalent) required to inhibit the cytopathicity of HIV-1 by 50% in MT-4 cells. [f] CC_{50}: concentration in μM (expressed in AZT equivalent) required to cause 50% death of uninfected MT-4 cells. [g] SI: selectivity index = CC_{50}/EC_{50}. [h] FIC: fractional inhibitory concentration. [i] Not calculable because the cytotoxicity of this conjugate is only due to its κ-carrageenan moiety at the concentration of 300 μg/mL.

a succinate diester linkage as mentioned above.[45] (vi) Interestingly, in NHS (pH 7.2), conjugate **3′** appeared to be more stable (only 39% of AZT and prodrug **1** are released from the macromolecular carrier) than prodrug **1** (100% of hydrolysis) (Table 1). These results (in contradiction with the concept of tripartite prodrugs via a succinate diester linkage, as demonstrated in Figure 2) suggest that the presence of the κ-carrageenan moiety protects the ester linkages from hydrolysis by the serum esterases. As has already been reported for curdlan sulfate,[62] polymeric drug delivery agents, because of their steric hindrance, can protect the ester bonds from hydrolysis within the active center of esterase. The total release of AZT (100%) in NHS from a free mixture of prodrug **1** and κ-carrageenan (data not shown) confirmed the effect of the steric hindrance observed when the two drugs are covalently bound. Another very interesting point, demonstrated through HPLC analysis during the hydrolysis of conjugate **3′** in NHS, is that the ester bond between the κ-carrageenan and the succinate spacer is 6-fold more stable than the ester bond between AZT and the succinate spacer.

In conclusion, the AZT release rate is prolonged in the case of conjugate **3′** in NHS compared with a prodrug without steric hindrance. This latter conjugate is stable at a neutral pH range in aqueous solutions and is hydrolyzed slowly through esterase catalysis in NHS to release free AZT and prodrug **1**, which is then hydrolyzed to AZT. These results suggest that κ-carrageenan may be expected to act as a suitable drug delivery agent (with intrinsic anti-HIV activity) giving a prolonged release of AZT.

Antiviral Activity Results. The different drugs were evaluated for their inhibitory effect on HIV-1 replication and their cytotoxicity in MT-4 cells (Tables 2 and 3). Two series of experiments were performed in order to compare the anti-HIV properties of prodrug **1**, conjugates **3** and **3′**, and the parent drugs (AZT and κ-carrageenan). In the first series, MT-4 cells were pretreated for 1 h with the drugs before HIV-1-infection (Table 2). In the second series, the drugs were added to the MT-4 cell cultures that had already been infected by HIV-1 (Table 3). The 50% effective concentration (EC_{50}) represents the concentration required to inhibit the virus-induced cytopathic effect by 50%.

(i) Effect of Pretreatment of MT-4 Cells with Antiviral Compounds on Anti-HIV Potency (Table 2). To estimate the contribution to anti-HIV activity (inhibition of virus attachment) from the κ-carrageenan moiety, we first administered the antiviral compounds before HIV-1-infection. As shown in Table 2, a three-and-one-half higher loading of AZT covalently bound onto κ-carrageenan resulted in a 40-fold (expressed in κ-carrageenan equivalent) or in an 11-fold (expressed in AZT equivalent) higher anti-HIV activity for conjugate **3′** (0.1 μg/mL or 6.8 nM, respectively) compared to conjugate **3** (4 μg/mL or 80 nM, respectively). Probably there is a critical ratio of the amount of the AZT covalently bound onto the carrier to the amount of κ-carrageenan to obtain optimal anti-HIV activity. In the present experiment, conjugate **3′** was found to be 100-fold more active (0.1 μg/mL) than κ-carrageenan (10 μg/mL) and 4 times more potent (6.8 nM) than AZT itself (25 nM).

(ii) Anti-HIV Potency of Antiviral Compounds on MT-4 Cells Already Infected by HIV-1 (Table 3). As shown in Table 3, conjugate **3′** was found to be seven-and-one-half-fold more active (1.6 μg/mL) than κ-carrageenan (12 μg/mL), while in contrast, AZT was 8 times more active than conjugate **3′** (expressed in AZT equivalent) in this experiment.

When the outcomes of both experiments i and ii are compared, pretreatment of MT-4 cells with conjugate **3′** before HIV-1-infection resulted in an increase of anti-

HIV potency (0.1 μg/mL with pretreatment compared to 1.6 μg/mL without pretreatment). These results suggest that preincubation of MT-4 cells with conjugate **3′** reduced virus entry into the cells. The κ-carrageenan−succinate diester−AZT conjugate **3′** inhibited the binding of the virions to the MT-4 cells and concomitantly delivered AZT to these cells (giving a prolonged release of AZT) to further inhibit the reverse transcription step during the HIV replicative cycle. In the case of conjugate **3′**, a synergistic anti-HIV activity occurred between the κ-carrageenan and AZT compounds. In fact, this synergistic effect is only observed when the cells are pretreated with conjugate **3′** before HIV-1-infection, as demonstrated below.

(iii) Synergistic Activity of κ-Carrageenan and AZT in MT-4 Cells.[63] EC_{50} values were used for calculation of the fractional inhibitory concentration (FIC), which allows one to demonstrate a synergistic effect between the two covalently linked drugs. The following formula allows one to calculate this value:

$$FIC = FIC_{\kappa-carrageenan} + FIC_{AZT}$$

in which

$FIC_{\kappa-carrageenan}$ = (EC_{50} of κ-carrageenan−AZT conjugate (expressed in κ-carrageenan equivalent))/(EC_{50} of κ-carrageenan alone)

and

FIC_{AZT} = (EC_{50} of κ-carrageenan−AZT conjugate (expressed in AZT equivalent))/(EC_{50} of AZT alone)

One should recall that depending on the FIC values calculated from the two participating drug moieties, the following conclusions can be drawn: when FIC < 0.5, there is a synergistic biological effect between the two drugs; when 0.5 < FIC < 1.0, there is a subsynergistic biological effect between the two drugs; when FIC = 1.0, there is an additive biological effect between the two drugs; when 1.0 < FIC < 2.0, there is subantagonistic biological effect between the two drugs; and when FIC > 2.0, there is an antagonist biological effect between the two drugs.[63,64]

As shown by the calculated FIC indexes in Tables 2 and 3, a synergistic anti-HIV activity was observed between κ-carrageenan and AZT only when MT-4 cells were pretreated with conjugate **3′** before HIV-1-infection (FIC = 0.28, Table 2). Under the same experimental conditions, mixtures of free κ-carrageenan and AZT in the same ratio as for the κ-carrageenan−AZT conjugates showed only additive effects (data not shown). Interestingly, conjugate **3**, which possessed a lower AZT loading than conjugate **3′**, did not show a synergistic anti-HIV activity (FIC = 3.60, in Table 2) after pretreatment of the MT-4 cells with this conjugate before HIV-1-infection. Probably there is a critical ratio between the AZT covalently bound onto the carrier and the κ-carrageenan amount that is needed to achieve an optimal anti-HIV response resulting in a synergistic activity.

(iv) Cytotoxicity and Selectivity Index. When the cytotoxicity (CC_{50}, 50% cytotoxic concentration, or concentration that causes 50% toxicity in MT-4 cells) of conjugates **3** and **3′** were considered, it appeared that these conjugates did not prove to be more toxic than κ-carrageenan itself. As shown by the results in Tables 2 and 3, the cytotoxicity of a conjugate is only due to its κ-carrageenan moiety. In fact, the cytotoxicity of the AZT moiety is masked by that of the κ-carrageenan moiety. The AZT loading onto the κ-carrageenan carrier is too weak for observation of the cytotoxicity due to AZT itself. Thus, a decreased EC_{50} (expressed in κ-carrageenan equivalent) reflected a gain in the selectivity index (SI) of the conjugates (Tables 2 and 3).

Conclusion

Here, we report, to our knowledge for the first time, the synthesis and anti-HIV properties of new covalently bound κ-carrageenan−AZT conjugates (tripartite prodrugs). Synergistic activity of the two drugs (being part of the covalent conjugate) was found when MT-4 cells were pretreated with conjugate **3′** before HIV-1 infection. However, a critical ratio of AZT bound onto κ-carrageenan is required to obtain a synergistic anti-HIV activity (Table 2). At the present time, investigations are underway in our laboratories in order to optimize the covalent coupling of AZT (or other nucleoside analogues) onto the κ-carrageenan carrier[55,57] and to select the best spacer between AZT and κ-carrageenan.[55,56,62] κ-Carrageenan−AZT conjugates may reduce the toxicity of AZT. At the present time, conjugate **3′** represents the hit compound of a new family of covalent κ-carrageenan−AZT conjugates that will require further studies for safety and anti-HIV efficacy.

Experimental Section

Nuclear magnetic resonance spectra (^1H and ^{13}C NMR) were recorded with a Brüker AC-250 spectrometer; chemical shifts are expressed as δ units (part per million) downfield from TMS (tetramethylsilane). Results from fast atom bombardment (FAB^+) mass spectral analysis were obtained by Dr. Astier (Laboratoire de Mesures Physiques-RMN, USTL, Montpellier, France) on a JEOL DX-100 using a cesium ion source and glycerol/thioglycerol (1:1) or *m*-nitrobenzyl alcohol (NOBA) as the matrix. Mass calibration was performed using cesium iodide. IR spectra were recorded on a Perkin-Elmer FTIR 1605 spectrophotometer. UV spectra were obtained with an Uvikon 930 spectrophotometer (Kontron Instrument). Microanalyses were carried out by Service Central d'Analyses du CNRS (Venaison, France) and were within 0.4% of the theoretical values. Analytical thin layer chromatography (TLC) experiments were performed using silica gel plates 0.2 mm thick ($60F_{254}$ Merck). Preparative flash column chromatography experiments were carried out on silica gel (230−240 mesh, G60 Merck). Analytical HPLC was performed on a Waters 600E instrument with a M991 detector using the following conditions: 4.6 mm × 150 mm column (Waters Spherisorb S5 ODS2, 5 μM); mobile phases A = 0.1% TFA in H_2O, B = CH_3OH; flow rate of 1 mL/min. All reagents were of commercial quality (Aldrich Company, Saint Quentin Fallavier, France) from freshly opened containers. κ-Carrageenan ($\overline{MW}$ = 600 000, $\overline{DPw}$ = 1470, I = 1.35; determined by size exclusion chromatography/multiangle laser light scattering (SEC−MALLS)) was obtained from SKW Biomaterials (l'Isle-sur-la-Sorgue, France). [2-^{14}C]-AZT (50 mCi/mmol) was purchased from Isotopchim (Peyruis, France). Radioactive counting in Emulsifier-Safe Packard was performed with a Packard 1600 TR 103656 liquid scintillation analyzer with internal chemiluminescence correction.

Cells and Viruses. (i) MT-4 Cells and HIV-1 (Strain BRU) (Table 2). The CEM cell line and the T-leukemia virus

type I (HTLV-I) $CD4^+$ T-cell line, MT-4, were cultured in RPMI/10% fetal calf serum (FCS), and the medium was replaced twice a week. The laboratory-adapted strain HIV clade B (strain BRU) stock was prepared from the supernatant of the infected CEM cell line, and aliquots were kept frozen at −80 °C until use.[1]

(ii) MT-4 Cells and HIV-1 (Strain III_B) (Table 3). MT-4 cells[65] were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 0.1% sodium bicarbonate, and gentamicin (20 μg/mL). The origin of the HIV-1 (strain III_B) stock has been described elsewhere.[66]

Anti-HIV Activity Assays. (i) Anti-HIV activity was monitored by the efficiency of the compounds to inhibit the cytopathicity of HIV-1 (strain BRU) in MT-4 cells (Table 2) as already described.[67,68] Briefly, 3×10^5 MT-4 cells were first preincubated with 100 μL of various concentrations of compounds dissolved in DMSO or in H_2O and then were diluted in phosphate-buffered saline (PBS) solution for 1 h at 37 °C. Then 100 μL of an appropriate virus dilution was added, and the cells were incubated for 1 h at 37 °C. After three washes, cells were resuspended in a culture medium in the presence or absence of the compounds. Cultures were then continued for 7 days at 37 °C under 5% CO_2 atmosphere, and the medium was replaced on day 3 postinfection with culture medium supplemented or not with drug compounds. Each culture condition was carried out in duplicate. Virus-induced cytopathicity was followed each day with an inverted optical microscope. Typically, the virus dilution used in this assay (multiplicity of infection of 0.1 CCID/cell) led to cytopathicity on day 5 postinfection. The inhibitory concentration of drug compounds was expressed as the concentration that caused 50% inhibition of viral cytopathicity (EC_{50}) without direct toxicity for the cells. The cytotoxic concentration (CC_{50}) of drug compounds was monitored on the basis of the growth of noninfected cells by the trypan blue exclusion method and corresponded to the concentration required to cause 50% cell death. It should be emphasized that when compounds required the addition of DMSO to be solubilized in water, the concentration in the volume of DMSO used was always less than 10% with respect to water (the final concentration of DMSO in MT-4 cells incubation medium being less than 2%). As far as DMSO could affect the antiviral activity of the tested compounds,[69] antiviral assays in which solutions containing equal concentrations of DMSO in water were performed and used as standard assays for each tested drug. EC_{50} and CC_{50} reported values were then calculated from these standard assays. Moreover, final DMSO dilutions ($^1/_{1000}$) were much lower than those enhancing in vitro HIV-1-infection of T-cells.[69]

(ii) The inhibitory effects of AZT, κ-carrageenan, and their prodrugs on HIV-1 (strain III_B) replication (Table 3) were monitored by the inhibition of the virus-induced cytopathicity in MT-4 cells 5 days after infection, as described elsewhere.[70] Cytotoxicity of the compounds was determined by measuring the viability of mock-infected cells on day 5.

Hydrolysis of Prodrug 1 in Buffers and in Human Serum (NHS).[56] To 990 μL of acetate buffer, water, PBS buffers, NaOH, or NHS was added a total of 10 μL of a solution of the desired prodrug **1** (10 mg/mL in DMSO), and the mixture was incubated at 37 °C in a water bath. At various time intervals, the samples (100 μL) were withdrawn and added immediately to ice-cold methanol (400 μL). The resulting samples were centrifuged (7 min, 3000 rpm). The supernatants were filtered through nylon filters (0.45 μm), and then analyzed by HPLC using the following method: 20% of solvent B in solvent A to 100% of solvent B in 20 min. The injection volume was 20 μL. The absorption maximum for the studied prodrug **1** was at 267 nm; therefore, this wavelength was used for the HPLC detection. Peak retention times (t_r) were 10.0 min for AZT and 14.1 min for compound **1**. The percentages of AZT release calculated from peak areas for compound **1** for hydrolysis in buffers and in NHS are summarized in Table 1.

Hydrolysis of Conjugate 3′ in Buffers and in Human Serum (NHS). To 1 mL of acetate buffer, water, PBS buffers, NaOH, or normal human serum (NHS) was added a total of 5 mg of the conjugate **3′**, and the mixture was incubated at 37 °C in a water bath. At various time intervals, the samples (150 μL) were withdrawn and added immediately to ice-cold methanol (350 μL). The resulting samples were centrifuged (7 min, 3000 rpm). A total of 300 μL of the supernatants was withdrawn, evaporated under reduced pressure, and diluted in 50 μL of methanol in order to obtain 6-fold concentrated samples. The supernatants were filtered through nylon filters (0.45 μm) and then analyzed by HPLC using the following method: 20% of solvent B in solvent A to 100% of solvent B in 20 min. The injection volume was 20 μL. The absorption maximum for the studied conjugate **3′** was at 267 nm; therefore, this wavelength was used for the HPLC detection. Peak retention times (t_r) were 10.0 min for AZT and 14.1 min for compound **1**. The percentages of AZT and compound **1** release calculated from peak areas for conjugate **3′** hydrolysis in buffers and in NHS are summarized in Table 1.

Chemical Syntheses. Mono(3′-azido-3′-deoxythymidin-5′-yl) Ester 1,4-Butanedioic Acid or 3′-Azido-3′-deoxy-5′-*O*-(succinate)thymidine 1. To a solution of AZT (0.802 g, 3.00 mmol, 1 equiv) in anhydrous DMF (15 mL), under N_2, containing DMAP (0.367 g, 3.00 mmol, 1 equiv), was added succinic anhydride (0.315 g, 3.15 mmol, 1.05 equiv). The reaction mixture was stirred at room temperature for 14 h. Then, the solvent was evaporated under reduced pressure. The residual oil was dissolved in CH_2Cl_2 (20 mL) and successively washed with 1 N HCl (2 × 15 mL) and water (2 × 15 mL). The combined aqueous solutions were extracted twice with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum. The residue was purified by flash chromatography on silica gel, using CH_3OH/CH_2Cl_2 8:92 as eluent, to give the title compound **1** as a white foam (0.937 g, 85%): $R_f = 0.22$ (CH_3OH/CH_2Cl_2 10:90); IR (CH_2Cl_2, cm^{-1}) 3482 (CO_2H), 3051 (NH), 2110 (N_3), 1720 and 1704 (broad C═O, acid and ester), 1690 (C═O, CONH); ^1H NMR (CD_3OD) δ 1.82 (s, 3H, CH_3Thy), 2.3−2.4 (m, 2H, H-2′,2″), 2.5−2.6 (m, 4H, AZT−$O_2C-CH_2-CH_2-CO_2H$), 3.9−4.0 (m, 1H, H-4′), 4.1−4.4 (m, 3H, H-3′, H-5′,5″), 6.08 (t, 1H, $J = 6.5$ Hz, H-1′), 7.44 (s, 1H, H-6); ^{13}C NMR (CD_3OD) δ 10.76 (CH_3Thy), 28.44 (AZT−$O_2C-CH_2-CH_2-CO_2H$), 28.52 (AZT−$O_2C-CH_2-CH_2-CO_2H$), 35.98 (C-2′), 60.03 (C-3′), 62.70 (C-5′), 81.33 (C-4′), 84.50 (C-1′), 110.13 (C-5), 135.97 (C-6), 150.34 (C-2), 164.46 (C-4), 172.27 (AZT−$O_2C-(CH_2)_2-CO_2H$), 175.41 (AZT−$O_2C-(CH_2)_2-CO_2H$); MS (FAB^+) 368 (M + H)$^+$; HPLC $t_r = 14.1$ min. Anal. ($C_{14}H_{17}N_5O_7$) C, H, N.

Mixed Anhydride of 3,4,5-Trimethoxybenzoic Acid and Mono-(3′-azido-3′-deoxythymidin-5′-yl) Ester 1,4-Butanedioic Acid 2. To a solution of the ω-carboxylic acid **1** (1.025 g, 2.79 mmol, 1 equiv) in anhydrous EtOAc (15 mL), under N_2, containing Et_3N (1.17 mL, 8.37 mmol, 3 equiv) was added dropwise at 0 °C 3,4,5-trimethoxybenzoyl chloride (0.708 g, 3.07 mmol, 1.1 equiv) in anhydrous EtOAc (10 mL). The reaction mixture was then stirred for 4 h at room temperature, diluted with EtOAc (15 mL), and washed twice with 0.5 N HCl (17 mL). After extraction, the organic layer was washed twice with water (2 × 20 mL) and dried over anhydrous Na_2SO_4. EtOAc was removed under reduced pressure, and the product slowly crystallized under vacuum to give the title compound **2** as a white powder (0.937 g, 85%). The resulting mixed anhydride was used in the next step without further purification: IR (CH_2Cl_2, cm^{-1}) 3020 (NH), 2111 (N_3), 1790 (C═O, anhydride), 1734 (C═O, ester), 1694 (C═O, CONH), 1590 (trimethoxybenzoyl), 1333 (OCH_3); ^1H NMR ($CDCl_3$) δ 1.84 (s, 3H, CH_3Thy), 2.3−2.4 (m, 2H, H-2′,2″), 2.64 (pt, 2H, AZT−$O_2C-CH_2-CH_2-CO_2CO-Ph(OCH_3)_3$), 2.73 (pt, 2H, AZT−$O_2C-CH_2-CH_2-CO_2CO-Ph(OCH_3)_3$), 3.82 (s, 6H, OCH_3 meta), 3.87 (s, 3H, OCH_3 para), 3.9−4.0 (m, 1H, H-4′), 4.1−4.4 (m, 3H, H-3′, H-5′,5″), 6.02 (t, 1H, $J = 6.2$ Hz, H-1′), 7.25 (s, 1H, H-6), 7.1−7.3 (pd, 2H, 2H ortho), 9.76 (brs, 1H, NH-3); ^{13}C NMR ($CDCl_3$) δ 12.73 (CH_3Thy), 28.64 (AZT−$O_2C-CH_2-CH_2-CO_2CO-Ph(OCH_3)_3$), 30.70 (AZT−$O_2C-CH_2-CH_2-CO_2CO-Ph(OCH_3)_3$), 37.60 (C-2′), 56.63 ($2H_3CO$ meta), 60.53 (C-3′), 61.27 (H_3CO para), 63.77 (C-5′), 81.96 (C-4′), 85.89 (C-1′),

108.21 (2C ortho), 111.56 (C-5), 123.76 (C ipso), 136.14 (C-6), 144.08 (C para), 150.50 (C-2), 153.51 (2C meta), 162.48 (AZT−O_2C−$(CH_2)_2$−CO_2CO−Ph$(OCH_3)_3$), 164.47 (C-4), 168.94 (AZT−O_2C−$(CH_2)_2$−CO_2CO−Ph$(OCH_3)_3$), 171.72 (AZT−O_2C−$(CH_2)_2$−CO_2CO−Ph$(OCH_3)_3$); MS (FAB$^+$) 562 $(M + H)^+$. Anal. ($C_{24}H_{27}N_5O_{11}$) C, H, N.

κ-Carrageenan−Succinate Diester−AZT Conjugates 3 and 3′. To a solution of κ-carrageenan (0.100 g, 0.245 mmol, 1 equiv for **3**; 0.113 g, 0.276 mmol, 1 equiv for **3′**; calculated on the repeating disaccharidic unit) in anhydrous DMF (15 or 18 mL for **3** or **3′**, respectively), under N_2, was added dropwise mixed anhydride **2** (0.172 g, 0.306 mmol, 1.25 equiv for **3**; 1.550 g, 2.760 mmol, 10 equiv for **3′**) in anhydrous DMF (10 mL). The reaction mixture was stirred at 60 °C for 72 h and then precipitated with 1-propanol (30 or 40 mL for **3** or **3′**, respectively), as is done for some polymers in solid-phase synthesis. The precipitate was filtered on a Büchner funnel and washed with acetone, methanol, dichloromethane, and acetone again to remove all the unbound products and the byproducts. The conjugates were dried under vacuum to give 82 mg of conjugate **3** and 92 mg of conjugate **3′**.

For conjugate **3**, $OD_{266} = 0.1952$, corresponding to $(2.0 \pm 0.5) \times 10^{-5}$ mmol of AZT covalently bound to 1 mg of κ-carrageenan.

For conjugate **3′**, $OD_{266} = 0.6608$, corresponding to $(6.8 \pm 0.5) \times 10^{-5}$ mmol of AZT covalently bound to 1 mg of κ-carrageenan.

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