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Regioselective synthesis of cyclodextrin mono-substituted conjugates of non-steroidal anti-inflammatory drugs at C-2 secondary hydroxyl by protease in non-aqueous media

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Abstract—Three β-cyclodextrin (β-CD) conjugates of non-steroidal anti-inflammatory drugs were synthesized by enzymatic methods. Transesterification of β-CD with vinyl ester of indomethacin, ketoprofen and etodolac was performed by the catalysis of alkaline protease from *Bacillus subtilis* in anhydrous DMF for 3 days. The drug molecules were selectively conjugated onto one of the secondary hydroxyl groups of β-CD through ester-linkage to improve their poor water solubility and absorption characteristics. The products were characterized by ESI-MS, ^{1}H NMR and ^{13}C NMR. The structures of products with monoacylation occurring at the C-2 secondary hydroxyl groups of β-CD were confirmed.

1. Introduction

Cyclodextrins (CDs) are truncated, cone-shaped cyclic oligosaccharides, consisting of six to eight glucose units through α-1,4 glucosidic bonds. Recently, cyclodextrins and various kinds of cyclodextrin derivatives have gained prominence in pharmaceutical field. One of the important applications is as a functional drug carrier to control the rate and/or the time profile of drug release and deliver the drug to targeted site.[1–3] Hydrophilic CDs can modify the release rate of poorly water-soluble drugs, which can be used for enhancement of drug absorption across biological barriers, serving as a potent drug carrier in the immediate release formulations.[4–6]

Indomethacin, ketoprofen and etodolac are some interesting non-steroidal anti-inflammatory drugs (NSAIDs), which possess poor water solubility, dissolution and low absorption characteristics. Considering the advantageous water solubility of cyclodextrin and their derivatives, preparation of CD complex of non-steroidal anti-inflammatory drugs is one useful approach to increase the solubility and bioavailability of NSAIDs.[7–9] However, the inclusion equilibrium of CD complex is sometimes disadvantageous for the degree of the dissociation being dependent on the magnitude of the stability constant of the complex. Especially drug targeting is to be attempted, because the complex would dissociate before it reaches the organ or tissues to which it is to be delivered.[1,10] One of the methods to prevent the dissociation is to bind a drug covalently to CD. Uekama and co-workers prepared the ketoprofen-α-cyclodextrin conjugate, which showed a typical delayed-release pattern in vitro and in vivo.[11] But in that case, protection/deprotection procedures and rigorous conditions were needed. Moreover, this cyclodextrin/drug conjugate approach can provide a versatile means for constructions of site-specific drug release system.[12,13]

Regioselective derivation of one hydroxyl group of cyclodextrin to prepare new derivative is an interesting but difficult challenge due to the large number of hydroxyl groups with similar reactivity. Generally, conventional chemical methods involve multi-step group transfer strategy and show an almost complete lack of regioselectivity.[14] The substrate such as drug is often covalently bound to several hydroxyl, thus inevitably leading to a mixture of mono-, di- and multi-substituted products.[15,16] For instance, Teranishi et al. reported the preparation of di-substituted cyclodextrin derivatives.[17,18] Hattori and Inazu synthesized glycol-cluster-modified cyclodextrins and dual recognition for lectin proteins and drugs.[19]

Keywords: Enzymatic synthesis; Regioselectivity; Cyclodextrin; Mono-substitution.
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Enzyme-catalyzed synthesis of sugar-based compounds has been proved to be a very efficient method due to the mild reaction conditions and high regioselectivity.[20–23] In our previous work, alkaline protease from *Bacillus subtilis* showed high regioselectivity in the esterification of carbohydrates in organic media.[24–26] Under the catalysis of subtilisin, vinyl-containing β-CD monoesters were synthesized by the transesterification of β-CD with divinyl dicarboxylates.[27]

In this paper, we wish to report the regioselectively enzymatic synthesis of β-CD conjugates of non-steroidal anti-inflammatory drugs. The alkaline protease from *B. subtilis* was used to catalyze the transesterification of β-CD with vinyl esters of indomethacin, ketoprofen and etodolac in non-aqueous medium, respectively. The drug molecules were selectively conjugated onto the secondary hydroxyl groups of β-CD through an ester-linkage and afforded mono-substituted ester type β-cyclodextrin conjugates of NSAIDs. These new derivatives are anticipated to be new candidates for controlled release and targeting prodrug.

2. Results and discussion

Protease received increasing attention for specific and regioselectively acylation of carbohydrates under very mild conditions. Efficient transesterification of β-CD with three vinyl esters of non-steroidal anti-inflammatory drugs was catalyzed by protease in DMF at 50 °C (Fig. 1). TLC and MS analysis showed that only mono-substituted esters were produced. The substitution position in compounds **3a**–**c** was confirmed by ^{1}H NMR and ^{13}C NMR.

Initial efforts to couple NSAIDs with β-CD focused on direct esterification of indomethacin, ketoprofen and etodolac with β-CD using enzyme methods. But no products were detected by TLC. The most probable reason is forming the by-product water, which is thermodynamically unfavourable to ester synthesis. Water removal is critical to achieve high yields in the enzymatic synthesis.[28] Moreover, the catalytic activity and stability of enzyme are negatively affected by higher concentration of water.[29] Thus we synthesize vinyl esters of indomethacin, ketoprofen and etodolac as more active acyl donors because the vinyl alcohol released tautomerizes to acetaldehyde.

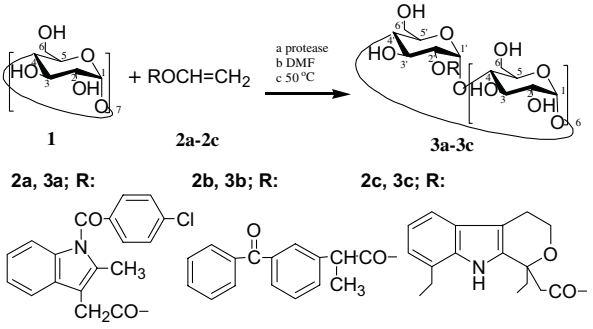

Figure 1. Enzymatic synthesis of β-CD conjugates of NSAIDs.

2.1. Mono-substituted β-CD NSAIDs conjugates catalyzed by protease

The degree of substitution (DS) for CD derivatives gives the average number of substituted groups per cyclodextrin molecule. The various methods for determining DS in cyclodextrins include elemental analysis, MS and ^{1}H and ^{13}C NMR spectrum. Because of the highly functionalized and symmetrical nature of CD, most CD derivatives prepared by conventional chemical catalysts are multi-substituted and involve different positional isomers. In a report of lipase catalyzed esterification of β-CD, as another approach for preparing CD derivatives, the DS of the CD derivatives was from 0.38 to 6.78 and the products were multi-substituted mixtures.[30] It is thus concluded that selective mono-substitution is not easy.

We have synthesized three polymerizable β-CD derivatives by alkaline protease from *B. subtilis*, 20% multi-substituted cyclodextrin derivatives were obtained in the case of excessive acyl donors in the transesterification of β-CD with divinyl dicarboxylates.[27] In this study, only mono-substituted products were obtained because of excess of β-CD. Moreover, divinyl dicarboxylates have higher reaction activity and smaller steric effect than the vinyl drug esters in the protease-catalyzed transesterifications.

In this work, the DS of compounds **3a**–**c** was determined by ^{1}H NMR and ESI-MS. TLC analysis showed that only one product was detected. Thus the yields of reactions should be close to the conversion. Mono-substitution of all three products was proved in ^{1}H NMR by the ratio of proton integral for drug portion and the glucose units of β-CD, and was also confirmed by positive ESI-MS. Molecular ions $[M+Na]^{+}$ at *m*/*z* 1497.4, 1379.4, 1427.5 corresponding to the molecular weight of **3a**,**b** and **c**, respectively.

2.2. Regioselective acylation of β-CD at C-2 secondary hydroxyls catalyzed by protease

The regioselective result was confirmed by ^{13}C NMR spectra as summarized in Table 1. Based on the general strategy described by Yoshimoto et al., acylation of a hydroxyl group would lead to the *O*-acylated carbon (*CH_2OCOR) downfield, while the adjacent carbon (*CCH_2OCOR) upfield in ^{13}C NMR.[31] Analysis of ^{13}C NMR spectra of all the products revealed that reactions occurred on the C-2 secondary hydroxyl position of β-CD. In the ^{13}C NMR spectrum of **3a**, a small peak around 74.23 ppm corresponding to the downfield shift of C-2′ from 72.75 ppm, and another small peak around 99.16 ppm corresponding to an upfield chemical shift of C-1′ from 102.5 ppm. Strong peaks around 72.75 and 102.5 ppm were signals of C-2 and C-1 located on the non-substituted glucose units, thus resembling the downfield shift of C-2 and the upfield shift of C-1 in 2-monotosyl-β-CD[32] and 2-monobenzoyl-β-CD **3d**[33] as listed in Table 1. Furthermore, as compared with C-6 substituted β-CD derivatives (e.g., 6-*O*-monobenzoyl-β-CD **3e** listed in Table 1),[34] no similar change was

Table 1. Chemical shifts of ^{13}C NMR of β-CD and its conjugates

Carbon numbers	β-CD D_2O	**3a** DMSO-d_6 + D_2O	**3b** DMSO-d_6 + D_2O	**3c** DMSO-d_6 + D_2O	**3d**[a] DMSO-d_6	**3e**[b] DMSO-d_6
C-1	102.58	102.69	102.46	102.46	101.98	102.69
		102.52	102.38	102.34		102.07
		102.42	102.13	102.20		102.01
		102.14	101.98			101.65
			101.88			
C-1′		99.16	98.67	98.87	98.29	
C-2	72.67	72.75	72.51	72.70	72.63	72.49
						72.28
C-2′		74.23	73.52	74.14	73.89	72.13
						72.02
C-3	73.89	73.67	73.86	73.53	72.99	73.28
		73.21	73.69			73.13
		72.91				
C-3′		70.05	69.70	70.75	69.62	73.09
						73.03
						72.94
C-4′	81.94	82.45	82.21	81.99	81.64	82.48
C-4		82.23	82.04	81.38	80.99	81.67
		81.93	81.93	81.07		81.60
		81.81	81.81	80.96		81.57
			81.42			81.34
			81.35			
C-5	72.89	72.32	72.15	72.56	72.05	72.49
C-5′		72.75	72.01	72.41	71.12	69.09
C-6	61.17	61.05	60.86	60.42	59.94	59.98
		60.57	60.43			59.72
			60.25			59.68
C-6′						64.26
Drug		171.67	196.15	169.30	165.92	165.70
		168.79	174.23	136.56		
		138.48	141.57	135.76		
		136.25	137.36	127.62		
		134.90	133.16	119.37		
		131.93	132.27	107.75		
		130.94	130.15	60.65		
		129.87	129.74	43.15		
		56.25	128.85	31.21		
		29.67	44.58	24.25		
		14.14	18.76	22.39		
				15.0		
				8.4		

[a] Compound **3d**, 2-*O*-monobenzoyl-β-CD, referred to by Hao et al.[33]
[b] Compound **3e**, 6-*O*-monobenzoyl-β-CD, referred to by Tong et al.[34]

observed in compounds **3a**–**c**. Meanwhile, in the ^{13}C DEPT spectrum of **3a**, only C-6 of cyclodextrin showed inverted signals at 60.5 ppm, but no obvious change of chemical shifts was observed in the DEPT spectrum. The evidence therefore suggests that the substitution occurred at C-2 secondary hydroxyl position.

The ^1H NMR spectra also provided substitutional information on the C-2 position. In the ^1H NMR spectrum of **3a**, the C-1 proton of one of the glucose residues appeared downfield as a separate doublet (δ 5.08 ppm) from the remaining six C-1 protons (multiplet δ 4.89 ppm), and a similar pattern was also observed in **3b** and **c**. This pattern was caused by the modification of the secondary hydroxyl group on C-2. However, in the spectra of 6-*O*-(2-hydroxypropyl)-β-CD derivatives[35] and 6-*O*-sulfoalkyl-β-CD derivatives,[36] all the seven C-1 protons appeared at 4.85 ppm as a single doublet, and no downfield separation occurred. Thus, the ^1H NMR result was consistent with that of ^{13}C NMR. β-CD has multiple hydroxyl groups on C-2, C-3 and C-6 positions of glucopyranose unit. Among these three kinds of hydroxyl groups, the secondary hydroxyl on C-3 are the least reactive, attributable to hydrogen bonds between the protons of the 3-hydroxyl groups and the oxygen atoms of the C-2 hydroxyl groups.[36] The hydroxyl groups on C-6 are the most reactive toward electrophilic reagents, because they are primary and the most basic (pK_a = 15–16). The secondary hydroxyls groups on the C-2 are the most acidic with pK_a of 12.1.[37] Generally, an electrophilic reagent attacks the C-6 position. However, in the transesterification catalyzed by alkaline protease from *B. subtilis*, the secondary hydroxyl on C-2 of CD is acylated preferentially over the primary hydroxyl groups.

Although the different structures of three non-steroidal anti-inflammatory drugs used in this research, the position acylated on β-CD was the same. This implies that the regioselectivity of the alkaline protease from *B. subtilis* is not affected by the structure of drug.

3. Conclusion

In conclusion, three β-cyclodextrin conjugates of indomethacin, ketoprofen and etodolac were synthesized using the alkaline protease from *B. subtilis* in DMF at 50 °C. In the transesterification with vinyl esters of indomethacin, ketoprofen and etodolac, the alkaline protease showed high regioselectivity and the obtained products were the 2-*O*-substituted β-CD monoesters. Further researches concerning the regioselectivity of the enzyme and investigation of dissolution and release behaviour of drug/β-CD conjugates are in progress.

4. Experimental

4.1. Materials

Alkaline protease from *B. subtilis* was purchased from Wuxi Enzyme Co. Ltd (Wuxi, PR China). β-Cyclodextrins were recrystallized twice in water and dried under 110 °C for 2 h. DMF was dried over 3 Å molecular sieves for 24 h prior to use. Vinyl esters of indomethacin, ketoprofen and etodolac were produced and purified as described in the literature.[38] Indomethacin, ketoprofen and etodolac were kindly provided by Taizhou Dongdong Pharmachem Co. Ltd (Taizhou, PR China). Vinyl acetate and all other chemicals were of the highest purity commercially available.

4.2. Analytical methods

The progress of reactions was monitored by TLC with an eluent consisting of ethyl acetate/methanol/water (10:8:2, by vol). The position of acylation in enzymatically prepared β-CD esters was established by ^{1}H NMR and ^{13}C NMR (Bruker AVANCE DMX 500). DMSO-d_6 and D_2O were used as solvents and TMS was used as an internal reference. Mass spectrometry data were obtained on Bruker Esquire-LC for electrospray (ESI-MS) measurements (solvent: methanol; positive mode).

4.3. Transesterification reactions

β-CD 4.5 g (4 mmol) was dissolved in 80 mL anhydrous DMF containing 2 mmol (0.77 g) vinyl indomethacin ester (**2a**), 0.56 g vinyl ketoprofen ester (**2b**) and 0.63 g vinyl etodolac ester (**2c**), respectively. The reaction was initiated by adding alkaline protease from *B. subtilis* (10 mg/mL) and the suspension was shaken at 250 rpm for 3 days at 50 °C. The enzyme-catalyzed transesterifications were determined by TLC (ethyl acetate/methanol/water = 10:8:2, by vol). The reaction was terminated by filtering off the enzyme and evaporation of DMF under reduced pressure. Crude products of **3a**–**c**, containing mono-substituted esters, minor solvent, unreacted CD and vinyl drug ester, were extracted by methanol for 3–5 times and filtrated. Because the product and vinyl drug esters can dissolve in methanol but β-CD is scarcely soluble in methanol, β-CD could be removed by simple filtration. Then the concentrated filtrate was isolated by silica gel chromatography with a gradient eluent consisting of ethyl acetate/methanol/water (10:5:1, by vol) to give compounds (**3a**–**c**).

4.4. Mono-(2-*O*-indomethacin)-β-cyclodextrin conjugate (3a)

Yellow powder (yield: 37%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O): δ (ppm): 7.64 (4H, m, Ar-H), 7.05 (2H, m, Ar-H), 6.74 (1H, m, ph-H), 5.10 (d, 1H, J = 3.3Hz, H-1′), 4.89 (m, 6H, H-1), 3.92 (m, 2H), 3.77 (5 H, m, –OCH_3, –$COCH_2$), 3.65–3.43 (br m, 40H, H-2, 3, 4, 5, 6), 2.19 (s, 3H, –CH_3); ^{13}C NMR (125 MHz, DMSO-d_6 + D_2O) is shown in Table 1 (**3a**); ESI-MS (m/z): 1497.4 $[M_1+Na]^+$, M_1 corresponding exactly to mono-(2-*O*-indomethacin)-β-CD conjugate's molecular weight.

4.5. Mono-(2-*O*-ketoprofen)-β-cyclodextrin conjugate (3b)

Yellow powder (yield: 31%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O): δ (ppm) 7.95–7.58 (m, 9H, Ar-H), 5.08 (d, 1H, J = 3.5 Hz, H-1′), 5.03–4.84 (m, 6H, H-1), 4.14 (m, 1H, –CH), 3.98 (m, 2H), 3.89–3.44 (br m, 40H, H-2, 3, 4, 5, 6), 1.54 (m, 3H, –CH_3); ^{13}C NMR (125 MHz, DMSO-d_6 + D_2O) is shown in Table 1 (**3b**); ESI-MS (m/z): 1394.4 $[M_2+Na]^+$, M_2 corresponding exactly to mono-(2-*O*-ketoprofen)-β-CD conjugate's molecular weight.

4.6. Mono-(2-*O*-etodolac)-β-cyclodextrin conjugate (3c)

Yellow powder (yield: 24%); ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O): δ (ppm) 7.21 (1H, d, J = 6.9 Hz, Ar-H), 6.89 (2H, m, Ar-H), 5.05 (d, 1H, J = 3.2 Hz, H-1′), 5.01–4.80 (m, 6H, H-1), 4.00–3.88 (m, 2H, –CH_2O), 3.84–3.44 (br m, 40H, H-2, 3, 4, 5, 6), 3.23–2.81 (br m, 6H, 3-CH_2), 1.83 (2H, m, –CH_2), 1.21 (3H, m, –CH_3), 0.80 (m, 3H, –CH_3); ^{13}C NMR (125 MHz, DMSO-d_6 + D_2O) is shown in Table 1 (**3c**); ESI-MS (m/z): 1427.5 $[M_3+Na]^+$, M_3 corresponding exactly to mono-(2-*O*-etodolac)-β-CD conjugate's molecular weight.

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Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.bmc.2005.03.031.

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