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N-2′-Acetoxybenzoyl Derivatives of Chitosan, *N*-Desulphated Heparin and 2-Amino-2-deoxy-D-glucose

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SUMMARY

N-2′-Acetoxybenzoyl (aspirin) derivatives (degree of substitution 0·35–1·00) of chitosan, N-desulphated heparin and 2-amino-2-deoxy-D-glucose were prepared by methods that gave yields in the range 65–86%. The salicylate of chitosan was isolated with a 98% yield. Aspirin or salicylic acid was released much more slowly from N-(2′-acetoxybenzoyl)-chitosan than from the salicylate of chitosan, and much faster at 37°C in 0·1 M NaOH solution than in 2% aqueous acetic acid solution. Salicylic acid was isolated from the dialysate (0·1 M NaOH solution) of N-(2′-acetoxybenzoyl)-chitosan.

INTRODUCTION

Chemically modified chitin and chitosan chains may have a number of novel applications. For example chitin and chitosan derivatives have been used as a polymer support for immobilising enzymes (Hirano & Miura, 1979; Muzzarelli, 1980; Yamaguchi *et al.*, 1982*a*) and for collecting metals (Hall & Yalpani, 1980; Muzzarelli, 1977; Sakaguchi *et al.*, 1981). A new class of polysaccharide gels can be formed by *N*-substitution of chitosan in aqueous acetic acid (Hirano *et al.*, 1976).

2-Acetoxybenzoic acid (aspirin) has analgesic, antipyretic and anti-inflammatory functions. Chitosan, *N*-desulphated heparin and 2-amino-2-deoxy-D-glucose may be used as carriers for drug delivery (Miyazaki

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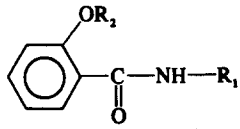

Derivative	R_1	R_2
1	Chitosan	Acetyl
2	Chitosan	H
3	2-Amino-2-deoxy-D-glucose	Acetyl and H
4	2-Amino-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucose	Acetyl
5	*N*-Desulphated heparin	Acetyl
6	Chitosan (salt)	H
7	2-Amino-2-deoxy-D-glucose (salt)	H

Fig. 1. Structure of derivatives investigated.

et al., 1981) because these molecules contain reactive amino groups which may easily be modified chemically. Aspirin derivatives of these molecules are interesting pharmaceutically, but the only reported work has been on 2′-acetoxy- and 2′-hydroxy-benzamido derivatives of 2-amino-2-deoxy-D-glucose and -D-galactose (Fujii & Kushida, 1967).

We now report the preparation and some properties of the aspirin derivatives of these molecules. The structure of the derivatives investigated is shown in Fig. 1.

MATERIALS AND METHODS

Chitosan, $[\alpha]_D^{17}$-10° (conc. 0·83, 2% acetic acid) was prepared from Flonac N (a commercial product of crab shell chitosan, Kyōwa Yushi Co. Ltd, Japan) as reported by Hirano & Kondo (1982). 2-Acetoxybenzoic anhydride was prepared by refluxing salicylic acid in acetic anhydride for three days (Nishi, 1967). *N*-Desulphated heparin was prepared from a commercial heparin from porcine intestinal mucosa (Sigma Chemical Company, St Louis, USA) (Hirano & Ohashi, 1977). Seamless cellulose tubing (Visking Company, USA) was used for the present dialysis experiments.

Infrared spectra were recorded with a Hitachi grating infrared spectrometer (215), ^{1}H-n.m.r. spectra with a Hitachi high resolution NMR spectrometer, and ultraviolet spectra with a Hitachi spectrophotometer (124). Specific rotations were measured with a Jasco digital polarimeter (Dip-181).

The other analytical methods have been cited previously (Hirano & Kondo, 1982).

N-(2'-Acetoxybenzoyl) chitosan (1)
A solution of chitosan (0·16 g) in 2% acetic acid (5 ml) was diluted with methanol (30 ml). To the solution was added 2-acetoxybenzoic anhydride (0·93 g, 3 mol/GlcN) dissolved in methanol (10 ml), and the mixture was stored at room temperature for 18 h, during which time the solution viscosity increased. The reaction mixture was concentrated under vacuum to a syrup, and ethanol (~30 ml) and ether (~10 ml) were successively added to give gelatinous precipitates. The precipitates were collected by filtration, washed with ether several times and dried over P_2O_5 *in vacuo* at 80°C for 5 h to give derivative 1 (0·20 g).

N-(2'-Hydroxybenzoyl) chitosan (2)
Derivative 1 (20 mg) was stirred in 0·01 M NaOH solution (20 ml) at room temperature for 2 h, and three volumes of ethanol was added to give derivative 2 (15 mg).

N-2'-Acetoxybenzoyl derivatives of 2-amino-2-deoxy-D-glucose 3 *and N-desulphated heparin* 5
Selective *N*-acylation was performed by each of the reported methods with 2-acetoxybenzoic anhydride in methanol to give 3 (Inouye *et al.*, 1956) and in formamide to give 5 (Hirano & Ohashi, 1977).

2-(2'-Acetoxybenzamido)-1,3,4,6-tetra-O-acetyl-2-deoxy-D-glucose (4)
Derivative 3 was peracetylated by the conventional method with acetic anhydride–pyridine (1:1, v/v) at room temperature for 18 h to give derivative 4.

Salicylate of chitosan (6)
Chitosan (0·16 g) was dissolved in an aqueous solution (60 ml) of aspirin (0·2 g) at room temperature by stirring for 3 h. The solution was concentrated under vacuum to a viscous solution, to which ethanol

and ether were added to afford precipitation. The precipitates were collected by filtration, washed with ether several times and dried over P_2O_5 *in vacuo* (0·30 g).

Salicylate of 2-amino-2-deoxy-D-glucose (**7**)
Aspirin was dissolved in a methanol solution of 2-amino-2-deoxy-D-glucose which was prepared from its hydrochloride and sodium in methanol (Inouye *et al.*, 1956). The solution was concentrated under vacuum to a small volume to give derivative **7**.

The physical constants and analytical data of the above derivatives are shown in Table 1.

Release of aspirin or salicylic acid through a dialysis membrane from N-*(2'-acetoxybenzoyl) chitosan* (**1**) *and salicylate of chitosan* (**6**)
Compounds **1** (50 mg) and **6** (34 mg) were dialysed through a dialysis membrane bag (~8 ml) against 2% aqueous acetic acid and 0·1 M NaOH solutions with mechanical stirring at 37°C. As a control experiment, aspirin (14 mg) was similarly treated against 0·1 M NaOH solution. The increase in absorption at 300 nm in the outer solution was analysed at appropriate time intervals. The release (%) of aspirin or salicylic acid from **1** and **6** through a dialysis membrane was expressed by (absorption in dialysate × 100)/(absorption of the total amount of salicylic acid present calculated from the formulae for **1** and **6**).

Detection of salicylic acid in the dialysate (0·1 M NaOH solution) of N-*(2'-acetoxybenzoyl) chitosan* (**1**)
The dialysate (0·1 M NaOH solution) of **1** was adjusted to pH ≅ 4 by addition of 6 M HCl solution, and the solution was concentrated by drying under vacuum. The salicylic acid released was extracted with ether (20 ml × 3 times), and the combined extracts were evaporated to give crystals, m.p. 155–158°C, λ_{max} (water) 298 nm. The infrared spectrum (KBr) and melting point were identical to that of salicylic acid.

RESULTS AND DISCUSSION

Chitosan, *N*-desulphated heparin and 2-amino-2-deoxy-D-glucose were successfully *N*-acylated by reaction of 2-acetoxybenzoic anhydride

TABLE 1

Physical Constants and Analytical Data on the Aspirin or Salicylic Acid Derivatives of Chitosan, *N*-Desulphated Heparin and 2-Amino-2-deoxy-D-Glucose

Derivative	*D.s. for aspirin*[a]	*Yield (%)*	$[\alpha]_D$ *(degree) (conc., solvent, temp. °C)*	λ_{max} *(nm)*[b]	*Formula*	*Calc. (%)*			*Found (%)*		
						C	*H*	*N*	*C*	*H*	*N*
1[c]	0·82	65	–	–	$[C_6H_{10}NO_4(C_9H_7O_3)_{0\cdot82}(H)_{0\cdot18}.0\cdot45H_2O]_n$	53·10	5·77	4·63	53·09	5·77	4·64
2[d]	0·70	81	−48(0·67, 0·01 M NaOH, 19)	330 (0·01 M NaOH)	$[C_6H_{10}NO_4(C_7H_5O_2)_{0\cdot70}(H)_{0\cdot30}.1\cdot62H_2O]_n$	47·71	6·26	5·11	47·70	6·35	5·09
3[e]	1·0	86	+38 (1·0, water, 19)	290 (water)				n.d.[f]			
4[g]	1·0	n.d.	+71 (0·37, chloroform, 28)	267,300 (ethanol)	$C_{23}H_{27}NO_{12}$	54·22	5·34	2·75	54·41	5·25	2·51
5	0·35[h]	76[i]	+33 (0·98, water, 18)	290 (water)				n.d.			
6[j]	0·91	98	+172 (1·1, water, 17)	295 (water)	$[C_6H_{11}NO_4(C_7H_6O_3)_{0\cdot91}.0\cdot43H_2O]_n$	58·21	6·84	5·49	57·98	6·87	5·36
7[k]	1·0	n.d.	+91 (0·74, water, 18)	297 (water)	$C_{13}H_{19}NO_8$	49·21	6·04	4·41	49·01	6·02	4·39

[a] Degree of substitution; the values are based on the C/N ratio in the elemental analyses, or on the ratio of the intensities of signals for (*o*-substituted phenyl protons)/(ring protons of the sugar) in the ^{1}H-n.m.r. spectra.

[b] λ_{max} 275 nm (water) for aspirin and 300 nm (water) for salicylic acid.

[c] ν_{max}^{KBr} ~3400 (OH, NH), 2900 (CH), 1740 and 1200 (C=O and C—O of *O*-acetyl), 1600 and 1480 (phenyl), 1630 and 1540 (C=O and NH of *N*-acyl), 1060–1000 (C—O—C), 900 (β-D) and 750 cm^{-1} (*o*-substituted phenyl).

[d] ν_{max}^{KBr} ~3400 (OH, NH), 2900 (CH), 1620 and 1540 (C=O and NH of *N*-acyl), 1600 and 1480 (phenyl), 1450, 1330, 1050 (C—O—C), 890 (β-D), and 760 cm^{-1} (*o*-substituted phenyl); ^{1}H-n.m.r. data (~0·1 M NaOD in D_2O): δ 8·8–6·8 (*o*-substituted phenyl protons) and 5·0–8·0 (ring protons of the sugar).

[e] ν_{max}^{KBr} ~ 3400 (OH, NH), 2900 (CH), 1750 and 1240 (C=O and C—O of *O*-acetyl), 1650 and 1440 (C=O and NH of *N*-acyl), 1610 and 1490 (phenyl), 1200, 1040 (C—O—C), and 750 cm^{-1} (*o*-substituted phenyl); ^{1}H-n.m.r. data (D_2O): δ ~7·30 (4 protons, *o*-substituted phenyl protons), 5·75–3·05 (6 protons, ring protons of the sugar) and 2·00 (1·5 protons, *O*-acetyl-methyl protons). The derivative was amorphous and hygroscopic, and was a mixture of 2′-acetoxybenzamido and 2′-hydroxybenzamido derivatives as revealed by the ratio of the intensities of signals for (acetyl-methyl protons)/(*o*-substituted phenyl protons). Literature (Fujii & Kushida, 1967) m.p. 124–128°C and $[\alpha]_D^{20}$ +47·5, water 37·3°C for the 2′-acetoxybenzamido derivative; m.p. 124–126°C and $[\alpha]_D^{20}$ +38·2, water 40·5°C for the 2′-hydroxybenzamido derivative.

[f] Not determined.

[g] Crystallised from ethanol, m.p. 216–218°C (uncorrected); ν_{max}^{KBr} ~ 3340 (NH), 2950 (CH), 1750 and 1230 (C=O and C—O of *O*-acetyl), 1670 and 1540 (C=O and NH of N-acyl), 1610 and 1490 (phenyl), 1020 (C—O—C), and 760 cm^{-1} (*o*-substituted phenyl); ^{1}H-n.m.r. data ($CDCl_3$): δ ~7·30 (*m*, 4 protons, *o*-substituted phenyl), 6·20 (*d*, 1 proton, H-1, $J_{1,2}$ 4·0 ppm), 5·4–3·8 (6 protons, ring protons of the sugar), and 2·30, 2·15, 2.06, 2·04 and 2·00 (*s*, 30 protons, acetyl-methyl protons).

[h] The d.s. value was calculated on the basis of the ratio of the intensities of signals for (*o*-substituted phenyl protons)/(ring protons of the sugar) in the ^{1}H-n.m.r. spectrum. ^{1}H-n.m.r. data (D_2O): δ 8·0–6·5 (*o*-substituted phenyl protons), 5·4–2·8 (ring protons of the sugars) and 2·15 (acetyl-methyl protons).

[i] Weight per cent on the basis of *N*-desulphated heparin.

[j] ν_{max}^{KBr} ~3400 (OH, NH), ~2700 (NH_3^+), 1630 and 1600 (CO_2^-), 1500, 1460, 1390, 1260, ~1100 (C—O—C), and 760 cm^{-1} (*o*-substituted phenyl). ^{1}H-n.m.r. data (D_2O): δ 8·0–6·5 (*o*-substituted phenyl protons), 5·5–3·0 (ring protons of the sugar), and no acetyl-methyl signals at δ ~ 2.

[k] ν_{max}^{KBr} ~3400 (OH, NH), 1620 and 1590 (CO_2^-), 1540, 1420, 1400, 1250, 1190, 1100, 1070, 1040, 100 (C—O—C), 920, and 770 (*o*-substituted phenyl).

in appropriate media (see Materials and Methods Section). *N*-(2′-Acetoxybenzoyl) chitosan (**1**), *N*-(2′-hydroxybenzoyl) chitosan (**2**), 2-deoxy-2-(2′-hydroxybenzamido)-D-glucose (**3**), *N*-(2′-acetoxybenzoyl) heparin (**5**) and the salicylate (2-hydroxybenzoate) of chitosan (**6**) were amorphous, while 2-(2′-acetoxybenzamido)1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-glucose (**4**) and the salicylate of 2-amino-2-deoxy-D-glucose (**7**) were crystalline. These aspirin and salicylic acid derivatives were isolated in yields in the range 65–98%. The physical constants and analytical data are given in Table 1. The *O*-deacetylation of 2′-acetoxybenzoyl residues was performed in 0·01 M NaOH solution at room temperature, but this treatment also resulted in partial *N*-deacylation. A partial *O*-deacetylation of *N*-2′-acetoxybenzoyl residues was observed with **3** and the complete *O*-deacetylation with **6** and **7** under the present conditions. This is probably due to the action of the basic free amino groups of the hexosaminide residues. Derivatives **3** and **5–7** were soluble in water and **2** was soluble in dilute alkaline solutions, but **1** and **4** were insoluble in water.

The presence of 2′-acetoxy- or hydroxy-benzoyl groups was detected by i.r. absorptions at 1740 and 1200 (C=O and C—O of *O*-acetyl) and 750 cm^{-1} (*o*-substituted phenyl) for these derivatives, and by ^{1}H-n.m.r. signals at δ 8·0–6·5 (*o*-substituted phenyl protons) and by u.v. absorptions at 267–330 nm for **2–7**. The degree of substitution (d.s.) values for aspirin or salicylic acid were calculated from the C/N ratios in the elemental analyses or from the ratio of the intensities of proton signals for (*o*-substituted phenyl protons)/(ring protons of the sugar) in the ^{1}H-n.m.r. spectra. The d.s. values were 0·70–0·91 for these chitosan derivatives.

As shown in Fig. 2, the free aspirin concentration in the 0·1 M NaOH dialysate reached equilibrium after 6 h. Aspirin or salicylic acid was released much more slowly from **1** than from **6**, and much faster in 0·1 M NaOH solution than in 2% aqueous acetic acid solution. Salicylic acid was isolated as crystals from the dialysate (0·1 M NaOH solution) of **1**. Therefore, *N*-2′-acetoxybenzoyl groups may be released from **1** by hydrolysis along with *O*-deacetylation of 2′-acetoxybenzoyl groups in alkaline solutions. The same reaction has been observed with *N*-formyl and *N*-trifluoroacetyl derivatives of chitosan, in which the *N*-acyl groups were hydrolysed in dilute alkaline solutions (Yamaguchi *et al.*, 1982*b*; Hirano & Kondo, 1982). It is interesting to note that *N*-2′-acetoxybenzoyl groups were slowly released even in only slightly

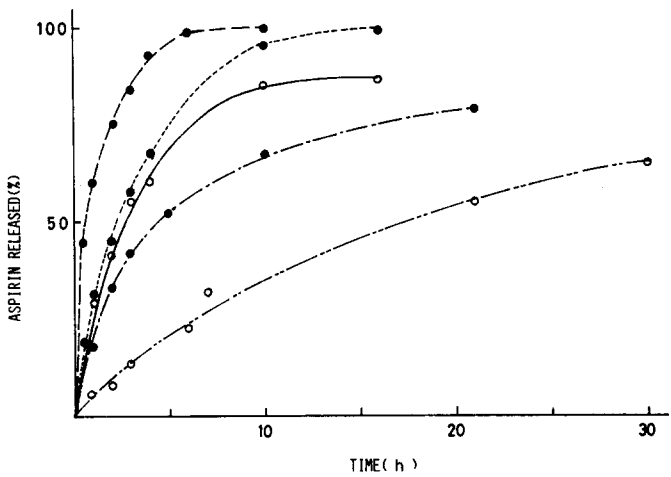

Fig. 2. Release of aspirin or salicylic acid through a dialysis membrane from *N*-(2'-acetoxybenzoyl) chitosan (**1**) and salicylate of chitosan (**6**) at 37°C in 0·1 M NaOH solution and in 2% aqueous acetic acid solution. Aspirin in 0·1 M NaOH solution (—●—); salicylate of chitosan in 0·1 M NaOH solution (--●--); *N*-(2'-acetoxybenzoyl) chitosan in 0·1 M NaOH solution (—·—●—·—); salicylate of chitosan in 2% aqueous acetic acid solution (—○—); *N*-(2'-acetoxybenzoyl) chitosan in 2% aqueous acetic acid solution (----○----). The release of aspirin or salicylic acid was calculated from (observed absorption at 300 nm in the dialysate x 100)/(absorption at 300 nm of total amount of salicylic acid present calculated from the formula).

acidic solutions. The release ratios (%) of aspirin or salicylic acid from these derivatives through a dialysis membrane for 6 h are compared with that (100% as salicylic acid in 0·1 M NaOH solution) of free aspirin: **6** was 80% in 0·1 M NaOH and 72% in 2% aqueous acetic acid, and **1** was 52% in 0·1 M NaOH and 20% in 2% aqueous acetic acid (Fig. 2).

Miyazaki *et al.* (1981) demonstrated a sustained release of indomethacin and papaverine dispersed in chitosan gels. Chitosan is a nontoxic biopolymer (Arai *et al.*, 1968) and this work suggests that it may be used as a carrier for drug delivery.

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