Synthesis of 1-β-*O*-acyl glucuronides of diclofenac, mefenamic acid and (*S*)-naproxen by the chemo-selective enzymatic removal of protecting groups from the corresponding methyl acetyl derivatives

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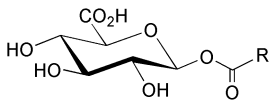
Using a straightforward chemo-enzymatic procedure, 1-β-*O*-acyl glucuronides of three non-steroidal anti-inflammatory drugs, diclofenac (DF) **5**, mefenamic acid (MF) **6** and (*S*)-naproxen (NP) **7**, were prepared. Caesium salts of these carboxylic acid drugs reacted with commercially available methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate **4** to give exclusively the corresponding 1-β-*O*-acyl glucuronides **8–10** in moderate yields. The protecting acetyl (for –OH group) and methyl ester (for $-CO_2H$ group) groups of each sugar moiety were easily removed to provide the corresponding free 1-β-*O*-acyl glucuronides **1–3** in high yields. Deprotection was achieved through effective enzyme-catalysed chemo-selective hydrolyses of the acetyl groups using lipase AS Amano (LAS), and of the methyl ester group using esterase from porcine liver (PLE).

Introduction

Much attention has been focused on the metabolic activation of drugs to reactive metabolites, on the covalent binding of these metabolites to target macromolecules (proteins and/or DNA), and on the toxicity of these metabolites under certain physiological conditions such as necrotic injury, idiosyncratic reaction and cancer.[1–7] 1-β-*O*-Acyl glucuronides are such chemically reactive metabolites, associated with carboxylic acids such as non-steroidal anti-inflammatory carboxylic acid drugs. Several isoforms of UDP-glucuronosyltransferase, which are responsible for the formation of these reactive 1-β-*O*-acyl glucuronides, have been identified.[8,9] 1-β-*O*-Acyl glucuronides are generally labile at physiological pH and are known not only to undergo ester-bond hydrolysis and intramolecular acyl migration, but also to bind covalently to proteins and other nucleophilic components.[10–15] It has been postulated that the covalent binding of 1-β-*O*-acyl glucuronides may disrupt the function of a critical protein and/or cause hypersensitivity. This possibility is based largely on the hapten hypothesis: some 1-β-*O*-acyl glucuronides are believed to be responsible for the adverse effects, such as hypersensitivity and cellular toxicity, of parent drugs.[11,12,16–18] Two mechanisms have been proposed to explain the covalent binding of 1-β-*O*-acyl glucuronides to proteins,[19,20] although the toxicological consequences of covalent binding remain ambiguous. 1-β-*O*-Acyl glucuronides have therefore attracted considerable toxicological and chemical interest regarding their structure–activity relationship.[21–25] The ability of 1-β-*O*-acyl glucuronides to bind to proteins is of particular interest for providing further insights into their intrinsic toxicities.



1-β-*O*-acyl glucuronide

Such toxicological and chemical studies require an efficient and widely applicable synthetic methodology for preparing pure 1-β-*O*-acyl glucuronides. To our knowledge, however, there is no convenient synthetic methodology to replace biosynthetic methods using liver microsomes fortified with UDPGA,[24,26] or the purification of 1-β-*O*-acyl glucuronides from urinary[27] and/or biliary[28] metabolites. Chemical synthetic methods reported to date can be divided into three categories based on the source of the glucuronosyl starting materials. The first method utilises benzyl 2,3,4-tri-*O*-benzylglucuronate,[29] with which carboxylic acids are conjugated primarily using either the Mitsunobu reaction[30] or trichloroacetimidate method.[31] The benzyl group is easily removed by hydrogenolysis without affecting the anomeric acyl functional group. This method, however, requires a multi-step process for preparing the starting benzylated glucuronate, and generally gives a mixture of anomers of 1-*O*-acyl glucuronide. The second method utilises unprotected glucuronic acid, which is acylated with *N*-acylimidazoles.[23,32] However, except for the specific case of a retinoylation reaction,[32] reported yields are relatively low. The third method utilises allyl glucuronate, which is acylated with carboxylic acids by the Mitsunobu reaction or the HATU–NMM procedure.[33,34] This approach produces the desired products with high β/α ratios, but the overall yields are unsatisfactory.

Herein we report a facile chemo-enzymatic approach for the preparation of 1-β-*O*-acyl glucuronides **1**, **2** and **3** from commercially available methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate **4** and the corresponding non-steroidal anti-inflammatory drugs (NSAIDs), namely: diclofenac (DF) **5**, mefenamic acid (MF) **6** and (*S*)-naproxen (NP) **7** (Fig. 1), respectively.

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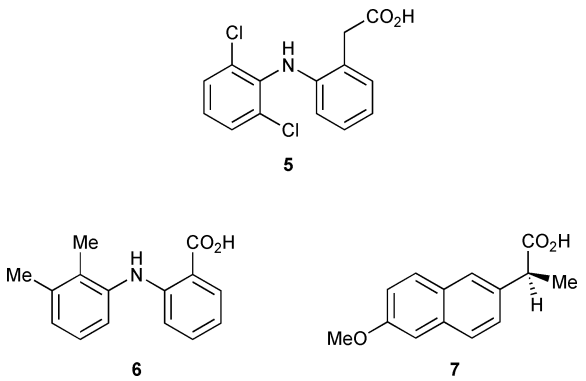


Fig. 1 Structures of NSAIDs used.

In the first glucuronidation step, methyl acetyl derivatives of 1-β-*O*-acyl glucuronides **8–10** were obtained easily and stereo-specifically. In the second step, enzymatic deprotection was performed with high chemo-selectivity using the commercially available lipase AS Amano (LAS) and esterase from porcine liver (PLE), as shown in Scheme 1.

Results and discussion

Synthesis of methyl acetyl derivatives of 1-β-*O*-acyl glucuronides 8–10

Complete anomeric stereocontrol is critical for the synthesis of 1-β-*O*-acyl glucuronides **1–3**. As indicated in reviews of carbohydrates,[35,36] the most reliable method for constructing 1,2-*trans*-glycosidic linkages is to utilise neighbouring-group participation from the 2-*O*-acyl functionality. Therefore, the glucuronidation of DF **5**, MF **6** and NP **7** was attempted using commercially available bromide **4**. Reactivity of the sodium salt of DF with **4** to provide **8** was first examined in several solvents (DMSO, DMF, diglyme, HMPA, 1,4-dioxane and MeOH) at 30 °C. DMSO was found to be the solvent of choice, whereas the reaction rate was very reduced in MeOH. Among the counter cations examined (sodium, caesium and tetraethylammonium), the caesium salt of DF in DMSO gave the best yield of the corresponding methyl acetyl derivative **8**. Thus, as shown in Scheme 1, treatment of **4** with the caesium salts of the carboxylic acids **5–7** (1.5 equivalents) in DMSO at 30 °C for 3 h gave the corresponding methyl acetyl derivatives **8–10** in moderate yields (Table 1).

^{1}H-NMR spectra (in $CDCl_3$) of these crude reaction products were obtained in order to check the product ratio of α- to β-anomers. ^{1}H-Chemical shifts of the crude products are also listed in Table 1; each anomeric proton was observed as a doublet at around δ 5.8, with J values of approximately 7.5 Hz. From their coupling constants, the stereochemistry of products **8–10** was assigned to the β-configuration.[37] No signal attributed to the formation of α-anomers was detected, indicating that this glucuronidation method gave exclusively the expected 1-β-*O*-acyl glucuronide derivatives.

Table 1 Yields of **8–10** and ^{1}H-NMR data of the anomeric protons of the crude products

NSAID	Product	Yield (%)	C_1-*H*/ppm[a] (*J*/Hz)
5	**8**	79	5.81 (7.6)
6	**9**	74	5.99 (7.3)
7	**10**	46	5.75 (7.6)

[a] ^{1}H-Chemical shifts of C_1-*H* (anomeric protons) are presented as δ values in $CDCl_3$.

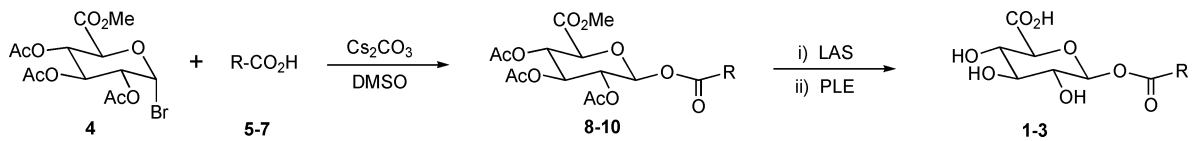
Similar stereo-selectivity, probably due to participation of the neighbouring 2-*O*-acetyl group, has been reported in the reactions of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide with caesium carboxylates[38] in DMF and with sodium carboxylates[39] in the presence of a phase transfer catalyst to yield 1-β-*O*-acyl-2,3,4,6-tetra-*O*-acetyl-D-glucopyranosides. In addition, bromide **4** has been reacted with a carboxylic acid[40] using Ag_2O. In this latter approach, the resulting methyl 1-β-*O*-acyl-2,3,4-tri-*O*-acetyl-D-glucopyranuronate was then chemically deacetylated, followed by an enzymatic hydrolysis of the methyl ester to give the corresponding free 1-β-*O*-acyl glucuronide in an overall yield of *ca.* 45%.[40]

Enzymatic deprotection of 8–10

The above reaction conditions yielded 1-β-*O*-acyl glucuronides **8–10** exclusively in moderate yields. The important issue of how to remove the protecting groups in these compounds without affecting their 1-β-*O*-acyl linkages remained. Recently, enzymes with high chemo-, regio- and/or stereo-selectivity have been employed as biocatalysts in organic reactions and many reviews of this research area have been published.[41]

Therefore, enzymes were screened in order to identify candidates capable of chemo-selectively hydrolysing the *O*-acetyl groups and/or the methyl ester group of **8–10**. Various enzymes including lipases and PLE were tested for the chemo-selective hydrolysis of **8** (chosen as the model substrate). Incubations were conducted using 0.5 mM **8** in 25 mM sodium citrate buffer (pH 5.0) containing 20% (v/v) DMSO as a co-solvent at 40 ± 0.1 °C for 30 min. Each commercially available enzyme was added to the incubation mixture at a final concentration of 5 mg cm^{-3}. The incubation was performed at pH 5.0 because the 1-β-*O*-acyl linkages of the corresponding partially and fully deprotected compounds derived from **8** were much less labile to both hydrolysis and intramolecular acyl migration at slightly acidic pH. Similar findings have been reported for other 1-β-*O*-acyl glucuronides.[15,42]

Of 11 enzymes tested, LAS and PLE showed hydrolytic activity toward **8**, whereas other enzymes did not show any hydrolytic activity toward **8** under the conditions used. LAS gave several



Scheme 1 Chemo-enzymatic synthesis of 1-β-*O*-acyl glucuronides.

major peaks on reversed-phase HPLC analysis, probably corresponding to partially and fully deacetylated compounds. After 4 h incubation, a major peak corresponding to methyl ester glucuronide **12** was detected, whereas PLE gave a major peak which was shown to correspond to tri-*O*-acetyl derivative **11**, as shown in Scheme 2. The hydrolytic activities of LAS and PLE were examined in more detail. The effect of co-solvents, including DMSO, on LAS-catalysed hydrolysis of **8** was first investigated at a concentration of 20% (v/v) in the incubation mixture. CH_3CN and tetrahydrofuran were ineffective at solubilising the substrate. 2-Methoxyethanol, DMF and *t*-BuOH were less effective in stimulating the hydrolytic reaction than DMSO. Fig. 2 shows chromatograms for the LAS-catalysed hydrolysis of **8** using DMSO as the co-solvent. The substrate (peak A, Fig. 2a) was fully deacetylated to provide methyl ester glucuronide **12** (peak D, Fig. 2b) after 5 h incubation; DF (peak E, Fig. 2b) was detected as a

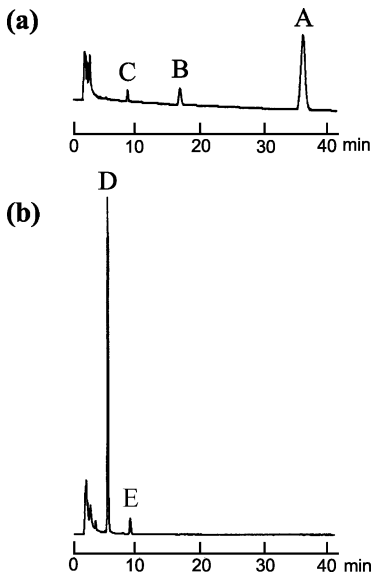
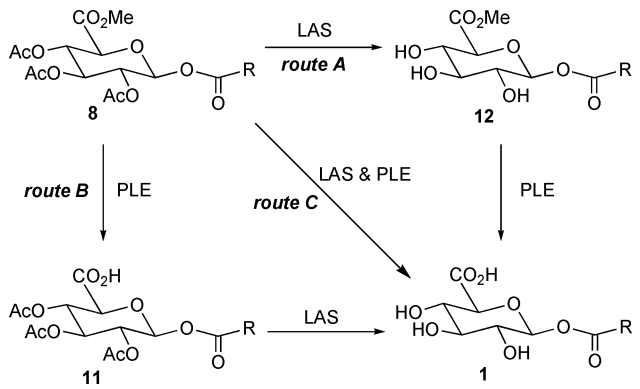


Fig. 2 HPLC chromatograms of LAS-catalysed hydrolysis of **8**. (a) after 2 min incubation; (b) after 5 h incubation. Conditions: 0.5 mM **8**, 20% (v/v) DMSO, 25 mM citrate buffer (pH 5.0), 8 mg cm^{-3} LAS and at 40 °C: A, **8**; D, **12**; E, **5**; B and C, partially deacetylated intermediates.



Scheme 2 Hydrolytic products of methyl acetyl derivative of diclofenac 1-β-*O*-acyl glucuronide (R-CO_2H = diclofenac) **1**. *Route A*, with LAS and then PLE; *route B*, with PLE and then LAS; *route C*, with both PLE and LAS.

Table 2 Reaction conditions and yields of LAS-catalysed hydrolyses of **8–10** to provide **12–14**

		Conditions	
Substrate	Yield (%)	pH (buffer)	Co-solvent (%, v/v [a])
8	94	5.0 (Ci[b]/NaOH)	DMSO (20)
9	90	6.0 (Pi[b]/NaOH)	DMSO (20)
10	90	5.5 (Ci–Pi[b]/NaOH)	2-Methoxyethanol (20)

[a] Percentage of the co-solvent in the incubation mixture. [b] Ci = citrate, Pi = phosphate, Ci–Pi = citrate–phosphate.

by-product at *ca.* 3% yield. These results indicated that DMSO was the best co-solvent of all those examined for the LAS-catalysed chemo-selective hydrolysis of **8** to **12**. Similarly, LAS-catalysed hydrolyses of **9** and **10** were performed under their respective optimum conditions to yield the corresponding methyl esters **13** and **14** in excellent yields. Reaction conditions and yields of LAS-catalysed hydrolytic products **12–14** are summarized in Table 2.

It is very important to choose a suitable co-solvent for LAS-catalysed hydrolysis in order to obtain high chemo-selectivity. For example, DMSO was a good co-solvent for the hydrolysis of **8** and **9**, but for **10** a considerable amount of NP (*ca.* 30%) was formed with the concomitant formation of the expected product **14**, probably due to lowered chemo-selectivity (data not shown). 2-Methoxyethanol was found to be the co-solvent of choice for the chemo-selective hydrolysis of the substrate **10**. These methyl esters **12–14** were easily purified by passage through an Amberlite XAD-4 column, followed by recrystallisation. The structures of these compounds were confirmed by ^{1}H- and ^{13}C-NMR, and by mass spectrometry.

PLE, commercially available as a mixture of isozymes,[43] is known to be one of the most useful enzymes for the synthesis of chiral synthons.[41] As mentioned above, PLE was shown to catalyse the hydrolysis of methyl ester of **8** to provide **11**. Therefore, PLE was examined for hydrolytic activity toward methyl esters **12–14** in order to obtain the free 1-β-*O*-acyl glucuronides **1–3**. In PLE-catalysed hydrolysis of the methyl ester **12** (chosen as the model compound), it is also very important to choose a suitable co-solvent to obtain high chemo-selectivity. For example, using DMSO as a co-solvent in the PLE-catalysed hydrolysis of **12** resulted in the formation of a considerable amount of DF (*ca.* 20%). When the co-solvent was changed to *t*-BuOH, the expected product **1** was obtained in 92% yield. Fig. 3a shows a typical HPLC chromatogram of the PLE-catalysed hydrolysis of **12** with *t*-BuOH as the co-solvent, where the corresponding free glucuronide **1** (peak A) was a major product and the hydrolysis generating DF (peak B) was suppressed almost completely. However, as shown in Table 3, DMSO was a good co-solvent for the PLE-catalysed hydrolysis of methyl esters **13** and **14**. The methyl ester groups of substrates **12–14** were chemo-selectively hydrolysed under specific conditions (pH and co-solvent) at 40 °C for 1 to 3 h, providing **1–3** in excellent yields (Table 3). In all cases, the incubation was performed at lower pH (between 5.0 and 6.0) rather than at the optimum pH for PLE (*ca.* 7 to 8) in order to prevent the non-enzymatic hydrolysis and intramolecular acyl migration of the 1-β-*O*-acyl linkages of both the substrates **12–14** and the products **1–3**.

Table 3 Reaction conditions and yields of PLE-catalysed hydrolyses of **12–14** to provide **1–3**

Substrate	Yield (%)	Conditions	
		pH (buffer)	Co-solvent (%, v/v[a])
12	92	5.0 (Ci[b]/NaOH)	*t*-BuOH (15)
13	99	6.0 (Pi[b]/NaOH)	DMSO (20)
14	90	5.5 (Ci–Pi[b]/NaOH)	DMSO (20)

[a] Percentage of the co-solvent in the incubation mixture. [b] Ci = citrate, Pi = phosphate, Ci–Pi = citrate–phosphate.

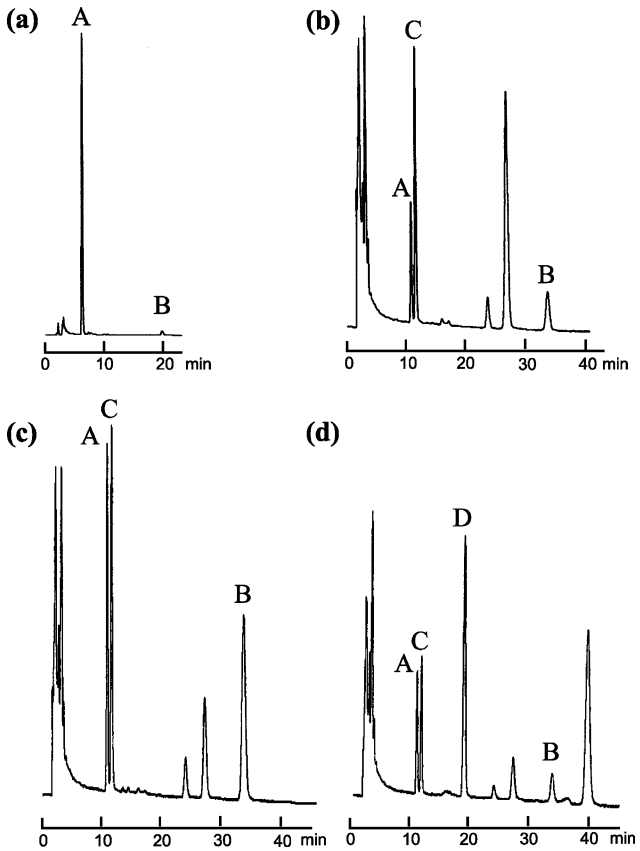


Fig. 3 HPLC chromatograms of (a) PLE-catalysed hydrolysis of **12**, after 4 h; (b) LAS-catalysed hydrolysis of **11** after 5 h; (c) both LAS- and PLE-catalysed hydrolysis of **8** with DMSO as a co-solvent, after 4 h; (d) both LAS- and PLE-catalysed hydrolysis of **8** with *t*-BuOH as a co-solvent after 4 h. A, **1**; B, **5**; C, a mono-*O*-acetyl derivative of **1**; D, **11**. Conditions: see Experimental.

Recently, selective hydrolysis of methyl and benzyl esters by microbial enzymes has been reported.[44] However, these reactions required over 24 h, and the enzymes' chemo-selectivity toward methyl and benzyl esters in the presence of acetoxy functional groups was not examined. Additionally, PLE has been also used for the hydrolysis[40] of a methyl ester of 1-β-*O*-acyl glucuronide at pH 7.0, but chemo-selectivity toward the substrate was not high. As shown in Fig. 4, PLE-catalysed hydrolysis of **8** proceeded smoothly within 4 h, with DF being only slightly formed. Our methodology using PLE at pH 5.0–6.0 has the advantages of high chemo-selectivity and a short reaction time.

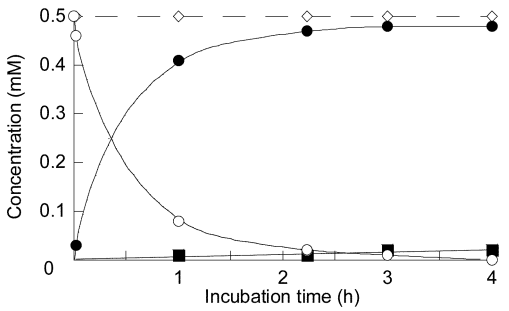


Fig. 4 Time course of PLE-catalysed hydrolysis of **12**. (○), **12**; (●), **1**; (■), DF; (◇), aggregate. Conditions: 0.5 mM **12**, 15% (v/v) *t*-BuOH, 0.4 mg cm^{-3} PLE at 40 °C.

The free 1-β-*O*-acyl glucuronides **1–3** were easily isolated by passage through an Amberlite XAD-4 column. The structures of these glucuronides were confirmed by ^{1}H- and ^{13}C-NMR, and by mass spectrometry. β-Glucuronidase is known to hydrolyse 1-β-*O*-acyl glucuronides but 2-, 3- or 4-*O*-acyl isomers formed through intramolecular acyl migration are resistant to hydrolysis by the enzyme.[12] Therefore, the purities of **1**, **2** and **3** as 1-β-*O*-acyl glucuronides were also determined using β-glucuronidase to be 97 ± 1, 99 ± 2 and 100 ± 1%, respectively. These results indicate that the glucuronides **1–3** are almost pure 1-β-*O*-acyl glucuronides.

The results presented here show that **8–10** are hydrolysed by both LAS and PLE; LAS hydrolyses the *O*-acetyl groups of **8–10** with high chemo-selectivity to provide **12–14** and PLE hydrolyses the methyl ester groups of **12–14** with high chemo-selectivity to provide **1–3**, *via* route A as shown in Scheme 2. As other possible routes, we examined a reverse combination of the enzymes (route B in Scheme 2) as well as the concurrent use of both enzymes (route C in Scheme 2). In route B, compound **11**, which is easily obtained by treatment of **8** with PLE though in an 'unsatisfactory yield', was incubated with LAS under the conditions used for the hydrolysis of **8** to **12**. As shown in Fig. 3b, the reaction did not go to completion and gave mixtures of **1** (peak A), **5** (peak B), a mono-*O*-acetyl derivative (peak C) and other unknown products. The ^{1}H-NMR spectrum of the mono-*O*-acetyl derivative showed a singlet peak at δ 1.92 corresponding to the –OCOCH_3 group. On the other hand, the concurrent use of both enzymes using DMSO and *t*-BuOH as the co-solvents resulted in improved results, as shown in Figs. 3c and 3d, respectively. In both the cases, other than the expected **1**, some hydrolysis of the 1-β-*O*-acyl linkage to provide DF (peak B) (especially remarkable in Fig. 3c) occurred and partially deacetylated intermediates (peak C corresponds to the mono-*O*-acetate) were accumulated. The data in Figs. 2b and 3b, showing LAS-catalysed hydrolysis of **8** and **11** respectively, indicated that LAS shows the high hydrolytic activity of *O*-acetyl groups in **8** but not in **11**. The difference of the activity might be due to the negative charge on the glucuronic acid moiety in the molecule of **11**.

From these results, the preferred sequential order for enzymatic treatment was shown to be LAS followed by PLE, namely the route A shown in Scheme 2. Application of this chemo-enzymatic method to the synthesis of other 1-β-*O*-acyl glucuronides, and screening of more efficient enzymes for the deprotection process, as well as optimisation of the enzymatic reaction conditions, are all currently under investigation.

Conclusions

A chemo-enzymatic synthesis of 1-β-*O*-acyl glucuronides was presented, taking DF **5**, MF **6** and NP **7** as examples for carboxylic acids. In the chemical glucuronidation step, methyl acetyl derivatives of 1-β-*O*-acyl glucuronides **8–10** were obtained exclusively in moderate yields, using neighboring 2-*O*-acetyl group participation. In the following enzymatic deprotection step, the expected free 1-β-*O*-acyl glucuronides **1–3** were easily obtained in excellent yields *via* a sequential LAS- and PLE-catalysed high chemo-selective hydrolysis.

Experimental

Materials

The sodium salt of DF **5** and free acids of MF **6** and NP **7** were obtained from MP Biomedicals, Lancaster and Wako Pure Chemical Industries, respectively. Methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate **4** was obtained from Sigma-Aldrich Co. or synthesised according to the reported procedure.[45] The 11 enzymes, used in the screening for chemo-selective hydrolytic activity, were: lipase AS Amano (from *Aspergillus niger*), lipase F-AP15 (from *Rhizopus oryzae*) and lipase G Amano 50 (from *Penicillium camemberti*), which were obtained from Wako Pure Chemical Industries, lipase from wheat germ and esterase from porcine liver (lyophilized powder), which were obtained from Sigma-Aldrich Co., lipase MY (from *Candida cyclindracea*), lipase OF (from *Candida cyclindracea*), lipase PL (from *Alcaligenes sp.*), lipase QLM (from *Alcaligenes sp.*), lipase SL (from *Pseudomonas cepacia*) and lipase TL (from *Pseudmonas stutzeri*), which were kindly gifted from Meito Sangyo Co., Ltd. Amberlite XAD-4 was obtained from ORGANO Corporation and used after grinding (80–200 mesh). All other chemicals used were analytical grade commercial products. ^{1}H- and ^{13}C-NMR were recorded using either a JNM-GX270 (JEOL) or a JNM-AL400 (JEOL). The chemical shifts measured in $CDCl_3$, d_6-DMSO or CD_3OD are presented by δ-values in ppm using the residual solvent peaks as internal standards relative to TMS.

Glucuronidation of 5–7 using methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate 4 to provide 8–10

A typical procedure is as follows: a solution of bromide **4** (397 mg, 1.00 mmol) and caesium salt of **5–7** (1.5 mmol) in DMSO (8 cm^3) was stirred at 30 °C for 3 h. The reaction mixture was diluted with EtOAc (100 cm^3) and then washed with water (100 cm^3), saturated aqueous $NaHCO_3$ (3 × 40 cm^3) and then saturated aq. NaCl. After drying over Na_2SO_4, the organic solvent was evaporated to give a crude product, which was purified by recrystallisation. Analytical and spectral data of the products **8–10** were as follows.

Methyl 2,3,4-tri-*O*-acetyl derivative of diclofenac 1-β-*O*-acyl glucuronide 8. Yield 79%; mp 155–157 °C (needles from EtOH). Found: C, 53.0; H, 4.5; N, 2.3; Cl, 11.5; *m/z* (FAB, positive) MNa^+, 634.0847. $C_{27}H_{27}Cl_2NO_{11}$ requires C, 52.95; H, 4.4; N, 2.3; Cl, 11.6%; $C_{27}H_{27}Cl_2NO_{11}Na$ requires *m/z* 634.0853; ν_{max} (KBr)/cm^{-1} 3360, 1750, 1510, 1460, 1370, 1230, 1090 and 1040; δ_H (270 MHz, d_6-DMSO) 1.82 (3H, s, OCOCH_3), 1.95 (3H, s, OCOCH_3), 1.99 (3H, s, OCOCH_3), 3.62 (3H, s, CO_2CH_3), 3.88 (2H, s, ArCH_2), 4.70 (1H, d, *J* 9.6, C_5-*H*), 4.99 (1H, t, *J* 8.2, C_2-*H*), 5.01 (1H, t, *J* 9.6, C_4-*H*), 5.50 (1H, t, *J* 9.6, C_3-*H*), 6.09 (1H, d, *J* 8.2, C_1-*H*), 6.22 (1H, d, *J* 7.9, Ar-*H*), 6.83 (1H, t, *J* 7.6, Ar-*H*), 6.98 (1H, s, N*H*), 7.05 (1H, t, *J* 7.6, Ar-*H*), 7.12–7.23 (2H, m, Ar-*H*) and 7.52 (2H, d, *J* 7.9, Ar-*H*); δ_C (67.8 MHz, d_6-DMSO) 20.03, 20.18, 20.24, 36.24, 52.58, 68.74, 69.81, 70.80, 71.41, 90.96, 115.83, 120.59, 122.09, 126.11, 127.95, 129.12, 130.80, 131.02, 137.04, 142.86, 166.86, 168.81, 169.25, 169. 39 and 169.62.

Methyl 2,3,4-tri-*O*-acetyl derivative of mefenamic acid 1-β-*O*-acyl glucuronide 9. Yield 74%; mp 124–125 °C (needles from EtOH). Found: C, 60.3; H, 5.5; N, 2.45; *m/z* (FAB, positive) MNa^+, 580.1801. $C_{28}H_{31}NO_{11}$ requires C, 60.3; H, 5.6; N, 2.5%; $C_{28}H_{31}NO_{11}Na$ requires *m/z* 580.1798; ν_{max} (KBr)/cm^{-1} 3340, 1760, 1700, 1610, 1580, 1510, 1460, 1380, 1230, 1100 and 1050; δ_H (400 MHz, d_6-DMSO) 2.00 (3H, s, OCOCH_3), 2.01 (3H, s, OCOCH_3), 2.01 (3H, s, OCOCH_3), 2.07 (3H, s, ArCH_3), 2.28 (3H, s, ArCH_3), 3.61 (3H, s, CO_2CH_3), 4.77 (1H, d, *J* 9.8, C_5-*H*), 5.11 (1H, t, *J* 9.5, C_4-*H*), 5.22 (1H, dd, *J* 7.8 and 9.5, C_2-*H*), 5.62 (1H, t, *J* 9.5, C_3-*H*), 6.25 (1H, d, *J* 7.8, C_1-*H*), 6.60 (1H, d, *J* 8.6, Ar-*H*), 6.72 (1H, m, Ar-*H*), 7.07–7.16 (3H, m, Ar-*H*), 7.36 (1H, dt, *J* 1.5 and 8.3, Ar-*H*), 7.78 (1H, dd, *J* 1.5 and 8.3, Ar-*H*) and 8.99 (1H, s, N*H*); δ_C (100 MHz, d_6-DMSO) 13.68, 20.15, 20.21, 20.28, 52.58, 68.80, 69.58, 70.44, 71.38, 90.04, 108.11, 113.42, 116.50, 123.28, 126.16, 127.20, 131.16, 132.10, 135.73, 137.63, 137.98, 149.64, 165.54, 167.00, 169.21, 169.29 and 169.41.

Methyl 2,3,4-tri-*O*-acetyl derivative of (*S*)-naproxen 1-β-*O*-acyl glucuronide 10. Yield 46%; mp 182–184 °C (needles from EtOH). Found: C, 59.39; H, 5.59; *m/z* (FAB, positive) MNa^+, 569.1615. $C_{27}H_{30}O_{12}$ requires C, 59.34; H, 5.53%; $C_{27}H_{30}O_{12}Na$ requires *m/z* 569.1625; ν_{max} (KBr)/cm^{-1} 1760, 1630, 1610, 1370, 1230, 1090 and 1040; δ_H (270 MHz, d_6-DMSO) 1.47 (3H, d, *J* 6.9, CHCH_3), 1.86 (3H, s, OCOCH_3), 1.95 (3H, s, OCOCH_3), 1.97 (3H, s, OCOCH_3), 3.59 (3H, s, CO_2CH_3), 3.86 (3H, s, ArOCH_3), 3.97 (1H, q, *J* 6.9, C*H*CH$_3$), 4.66 (1H, d, *J* 9.9, C_5-*H*), 4.95–5.02 (2H, m, C_2-*H* and C_4-*H*), 5.50 (1H, t, *J* 9.6, C_3-*H*), 6.05 (1H, d, *J* 8.2, C_1-*H*), 7.16 (1H, dd, *J* 2.6 and 9.2, Ar-*H*), 7.30 (1H, d, *J* 2.6, Ar-*H*), 7.35 (1H, dd, *J* 1.6 and 8.6, Ar-*H*), 7.69 (1H, s, Ar-*H*) and 7.78 (2H, d, *J* 8.9, Ar-*H*); δ_C (67.5 MHz, d_6-DMSO) 17.94, 20.08, 20.14, 20.24, 44.19, 52.56, 55.14, 68.78, 69.71, 70.75, 71.38, 90.93, 105.69, 118.81, 125.88, 126.15, 127.00, 128.29, 129.16, 133.43, 134.27, 157.28, 166.81, 168.86, 169. 21, 169.39 and 172.09.

HPLC analysis

HPLC was performed with a Shimadzu LC-10A equipped with a C-R8A chromatopac, using a column of Symmetry C_{18} (5 μm, 4.6 × 150 mm, Waters) for the analysis of the enzymatic reactions. Mobile phases for the analytical HPLC are given in each experiment described below. For the final purification of free 1-β-*O*-acyl glucuronides **1–3**, a semipreparative column of Symmetry C_{18} (7 μm, 19 × 150 mm, Waters) was used and the mobile phase for **1–3** was aqueous CH_3CN containing 0.01% (v/v) AcOH at a flow rate of 3 cm^3 min^{-1}. The concentrations of CH_3CN were 40, 50 and 30% (v/v) for **1**, **2** and **3**, respectively.

Screening of enzymes for the hydrolytic activity of 8 and 12 as model substrates

The incubation was carried out at an initial concentration of 0.5 mM of **8** or **12** in 25 mM sodium citrate buffer (pH 5.0) containing 20% (v/v) DMSO as a co-solvent at 40 ± 0.1 °C for 30 min and each commercially available enzyme was added to the incubation mixture at a final concentration of 5 mg cm^{-3} (except for PLE at 0.4 mg cm^{-3}). For enzyme assays, an aliquot of the incubation mixture was appropriately diluted with an HPLC carrier and injected onto a reversed-phase HPLC. The mobile phase of HPLC analysis for the hydrolysis of **8** was 55% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM tetra-*n*-butylammonium bromide (TBAB) at a flow rate of 0.7 cm^3 min^{-1} with detection at 283 nm. For analysis of the formation of **1** from **12**, the concentration of CH_3CN in the mobile phase was reduced to 45% (v/v). By adding TBAB (at a final concentration of 10 mM) to the mobile phase, polar 1-β-*O*-acyl glucuronides **1–3** could be separated from the other, more polar components derived from the enzymes, owing to the retardation of the retention times of **1–3**. This is explained by the formation of less polar ion pairs between the glucuronides and the quaternary ammonium ion.

Screening of co-solvents for the hydrolysis of 8 or 12

In order to optimise the LAS- and PLE-catalysed chemo-selective hydrolytic conditions, the effect of co-solvents (at the fixed final concentration of 20% (v/v)) on the enzymatic activity was examined with substrate **8** for LAS and **12** for PLE.

PLE-catalysed hydrolysis of 8 to 11 (route B)

A solution of **8** (77 mg, 0.126 mmol) in CH_3CN (37 cm^3) was added to 213 cm^3 of 25 mM sodium citrate buffer (pH 5.0) PLE (135 mg, 0.54 mg cm^{-3} incubation mixture) was dissolved at 40 °C and the mixture was incubated for 6 h. The mobile phase of HPLC analysis for this reaction was 55% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM TBAB at a flow rate of 0.7 cm^3 min^{-1} with detection at 283 nm. After the reaction mixture was evaporated to remove CH_3CN, the residual aqueous solution was acidified to pH *ca.* 3 and saturated with NaCl and then extracted with EtOAc (2 × 100 cm^3). After drying over Na_2SO_4, the organic solvent was evaporated to give a syrup, which was purified by silica gel pTLC (EtOAc–acetone–AcOH = 10 : 10 : 0.2, v/v) to provide **11** as a solid (40 mg, 53%); *m/z* (FAB, positive) MNa^+, 620.0692. $C_{26}H_{25}Cl_2NO_{11}Na$ requires *m/z* 620.0697; δ_H (400 MHz, CD_3OD) 1.76 (3H, s, $OCOCH_3$), 1.96 (3H, s, $OCOCH_3$), 1.99 (3H, s, $OCOCH_3$), 3.83 (1H, d, *J* 15.1, $ArCH_2$), 3.88 (1H, d, *J* 15.1, $ArCH_2$), 4.23 (1H, d, *J* 9.8, C_5-*H*), 5.11 (1H, t, *J* 9.3, C_4-*H*), 5.20 (1H, t, *J* 9.8, C_2-*H*), 5.35 (1H, t, *J* 9.5, C_3-*H*), 5.91 (1H, d, *J* 8.1, C_1-*H*), 6.40 (1H, d, *J* 8.1, Ar-*H*), 6.91 (1H, t, *J* 7.6, Ar-*H*), 7.02 (1H, s, N*H*), 7.06–7.10 (2H, m, Ar-*H*), 7.19 (1H, d, *J* 7.6, Ar-*H*) and 7.40 (2H, d, *J* 7.9, Ar-*H*); δ_C (67.5 MHz, CD_3OD) 20.31, 20.51, 20.59, 38.55, 70.83, 71.70, 73.69, 74.79, 93.09, 118.80, 122.98, 124.85, 126.03, 129.20, 130.06, 131.51, 132.06, 139.10, 144.26, 170.85, 171.15, 171.43 and 171.80.

LAS-catalysed hydrolysis of 11 (route B); isolation of the mono-*O*-acetyl derivative of 1

A solution of tri-*O*-acetyl derivative **11** (20 mg, 0.033 mmol) in DMSO (3.5 cm^3) was added to 14.5 cm^3 of 25 mM sodium citrate buffer (pH 5.0), LAS (270 mg, 15 mg cm^{-3} incubation mixture) was dissolved at 40 °C and the mixture was incubated for 5 h. The mobile phase of HPLC analysis for this reaction was 45% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM TBAB at a flow rate of 0.7 cm^3 min^{-1} with detection at 283 nm. After incubation for 3 h, the substrate **11** was almost hydrolysed to give a mixture. The amount of expected free glucuronide **1** for the 5 h incubation was almost same as that for the 3 h incubation (see Fig. 3b). A product whose retention time was very close to that of **1** was purified by pTLC ($CHCl_3$–MeOH–AcOH = 25 : 9 : 1, v/v) to give a 3- or 4-mono-*O*-acetyl derivative of **1** (4.0 mg, 23%), whose structure was estimated from ^{1}H-NMR; δ_H (270 MHz, d_6-DMSO) 1.92 (3H, s, $OCOCH_3$), 3.28–3.60 (3H, m, sugar-*H*), 3.84 and 3.92 (each 1H, d, *J* 15.8, $ArCH_2$), 4.78 (1H, t, *J* 9.6, C_3- or C_4-*H*), 5.43 (1H, d, *J* 7.6, C_1-*H*), 6.84 (1H, t, *J* 6.6, Ar-*H*), 7.07 (2H, t, *J* 8.9, Ar-*H*), 7.16–7.24 (2H, m, Ar-*H*) and 7.53 (2H, d, *J* 7.9, Ar-*H*). The proton at δ 4.78 was assigned to the *H*-C-$OCOCH_3$ proton of the sugar moiety due to its resonance at lower field. Its triplet multiplicity showed the proton to be either C_3- or C_4-*H*.

Concurrent usage of LAS and PLE for the hydrolysis of 12 (route C)

A solution of **8** in either 20% (v/v) DMSO or 15% (v/v) *t*-BuOH was added to 25 mM sodium citrate buffer (pH 5.0), LAS (15 mg cm^{-3} incubation mixture) and PLE (0.4 mg cm^{-3} incubation mixture) were dissolved at 40 °C and the mixture was incubated for 5 h. The mobile phase of HPLC analysis for this reaction was 45% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM TBAB at a flow rate of 0.7 cm^3 min^{-1} with detection at 283 nm. As shown in Figs. 3c and 3d, both the enzymatic reactions did not give good results; yields of the free glucuronide **1** after 4 h incubation with DMSO and *t*-BuOH as co-solvents, determined by HPLC, were *ca.* 30 and 10%, respectively.

LAS-catalysed hydrolysis of 8–10 to 12–14 (route A)

Methyl ester of diclofenac 1-β-*O*-acyl glucuronide 12. Incubation was started by the addition of a solution of **8** (50 mg, 0.082 mmol) in DMSO (32 cm^3) to 128 cm^3 of 25 mM sodium citrate buffer (pH 5.0), LAS (1.6 g, 10 mg cm^{-3} incubation mixture) was dissolved at 40 °C and the mixture stirred magnetically for 3 h. The conversion yield to **12** was 94% by HPLC analysis. The reaction mixture was then filtered and the filtrate was loaded on an Amberlite XAD-4 column (5 g), which had been washed thoroughly with acetone and then equilibrated with water. The column was washed with water (50 cm^3) and then 30% CH_3CN (50 cm^3). The hydrolysed product **12** was eluted with 80% CH_3CN (50 cm^3) with a recovery yield of 97% (by HPLC analysis). The solvent was evaporated to provide **12**. A portion of the sample was purified by recrystallisation from CH_3CN to confirm the structure by spectral analyses. *m/z* (FAB, positive) MNa^+, 508.0538. $C_{21}H_{21}Cl_2NO_8Na$ requires *m/z* 508.0540; δ_H (400 MHz, d_6-DMSO) 3.21–3.39 (3H, m, C_2-, C_3- and C_4-*H*), 3.64 (3H, s, CO_2CH_3), 3.82–3.93 (3H, m, $ArCH_2$ and C_5-*H*), 5.28 (1H, d,

J 4.9, O*H*, exchangeable with D_2O), 5.41 (1H, d, *J* 5.6, O*H*, exchangeable with D_2O), 5.43 (1H, d, *J* 4.9, O*H*, exchangeable with D_2O), 5.50 (1H, d, *J* 8.1, C_1-*H*), 6.22 (1H, d, *J* 8.1, Ar-*H*), 6.84 (1H, t, *J* 7.6, Ar-*H*), 6.93 (1H, s, N*H*), 7.06 (1H, t, *J* 7.6, Ar-*H*), 7.21 (2H, t, *J* 7.8, Ar-*H*), and 7.52 (2H, d, *J* 8.3, Ar-*H*); δ_C (100 MHz, d_6-DMSO) 36.61, 51.96, 71.21, 72.17, 75.18, 75.82, 94.59, 115.65, 120.51, 122.31, 126.15, 127.90, 129.11, 131.01, 131.04, 136.99, 142.90, 168.80 and 170.32.

Methyl ester of mefenamic acid 1-β-*O*-acyl glucuronide 13. Incubation was started by the addition of a solution of **9** (40 mg, 0.072 mmol) in DMSO (48 cm^3) to 144 cm^3 of 25 mM sodium phosphate buffer (pH 6.0), LAS (3.6 g, 15 mg cm^{-3} incubation mixture) was dissolved at 40 °C and the mixture stirred magnetically for 5 h. The conversion yield to **13** was 90% by HPLC analysis; the mobile phase for HPLC analysis was 60% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM TBAB and the detection was at 283 nm. The reaction mixture was then filtered and the filtrate was loaded on an Amberlite XAD-4 column (5 g), which was prepared as described for the purification of **12**. The column was washed with water (50 cm^3) and then 40% CH_3CN (50 cm^3). The hydrolysed product **13** was almost quantitatively recovered by elution with 90% CH_3CN (50 cm^3). The solvent was evaporated to provide **13**. A portion of the sample was purified by recrystallisation from CH_3CN to confirm the structure by spectral analyses. δ_H (270 MHz, CD_3OD) 2.13 (3H, s, ArCH_3), 2.31 (3H, s, ArCH_3), 3.67–3.60 (3H, m, C_2-, C_3- and C_4-*H*), 3.76 (3H, s, CO_2CH_3), 4.04 (1H, d, *J* 9.6, C_5-*H*), 5.76 (1H, d, *J* 7.6, C_1-*H*), 6.69–6.59 (2H, m, Ar-*H*), 7.06–7.11 (3H, m, Ar-*H*), 7.27 (1H, t, *J* 7.3, Ar-*H*), 8.04 (1H, d, *J* 8.2, Ar-*H*), 9.10 (1H, s, N*H*); δ_C (67.5 MHz, d_6-DMSO) 14.09, 20.60, 52.92, 72.96, 73.66, 77.38, 95.65, 110.62, 114.50, 117.19, 124.78, 127.22, 128.37, 132.93, 133.87, 136.08, 139.45, 151.54, 151.70, 168.33, 170.85.

Methyl ester of (*S*)-naproxen 1-β-*O*-acyl glucuronide 14. Incubation was started by the addition of a solution of **10** (44 mg, 0.081 mmol) in 2-methoxyethanol (32 cm^3) to 128 cm^3 of 25 mM sodium citrate and phosphate buffer (pH 5.5), LAS (2.4 g, 15 mg cm^{-3} incubation mixture) was dissolved at 40 °C and the mixture stirred magnetically for 2 h. The conversion yield to **14** was 90% by HPLC analysis; the mobile phase for HPLC analysis was 50% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM TBAB and the detection was at 271 nm. The reaction mixture was then filtered and the filtrate was loaded on an Amberlite XAD-4 column (5 g), which was prepared as described for the purification of **12**. The column was washed with water (50 cm^3) and then 40% CH_3CN (50 cm^3). The hydrolysed product **14** was almost quantitatively recovered by elution with 90% CH_3CN (50 cm^3). The solvent was evaporated to provide **14**. A portion of the sample was purified by recrystallisation from CH_3CN to confirm the structure by spectral analyses. δ_H (270 MHz, CD_3OD) 1.55 (3H, d, *J* 7.3, CHCH_3), 3.39–3.52 (3H, m, C_2-, C_3- and C_4-*H*), 3.69 (3H, s, CO_2CH_3), 3.88–3.98 (5H, m, C_5-*H*, C*H*CH_3 and ArOCH_3), 5.52 (1H, d, *J* 7.9, C_1-*H*), 7.09 (1H, dd, *J* 2.6 and 8.9, Ar-*H*), 7.18 (1H, d, *J* 2.0, Ar-*H*), 7.37 (1H, dd, *J* 2.0 and 8.6, Ar-*H*) and 7.66–7.72 (3H, m, Ar-*H*); δ_C (67.5 MHz, CD_3OD) 19.16, 46.39, 52.86, 55.71, 72.88, 73.53, 77.32, 95.84, 106.55, 119.91, 127.13, 127.23, 128.26, 130.25, 130.39, 135.28, 136.49, 159.18, 170.79 and 174.88.

PLE-catalysed hydrolysis of 12–14 to 1–3 (route A)

Diclofenac 1-β-*O*-acyl glucuronide 1. The methyl ester **12** (46 mg, 95 μmol) was dissolved in *t*-BuOH (28 cm^3) and the solution was poured into 123 cm^3 of 25 mM sodium citrate buffer (pH 5.0). To the resultant solution, PLE (76 mg, 0.4 mg cm^{-3} incubation mixture) dissolved in an aqueous solution (38 cm^3) was added and incubated at 40 °C for 4 h. The conversion yield to **1** was 92% by HPLC analysis (the mobile phase is described above). The reaction mixture was then acidified (to pH *ca.* 3) with 6 M HCl and then filtered off. The filtrate was loaded on an Amberlite XAD-4 column (5 g), which was prepared as described above. The column was washed with water (100 cm^3) and then eluted with 30% CH_3CN (50 cm^3) and 80% CH_3CN (50 cm^3). Both aqueous CH_3CN fractions, containing **1**, were combined and evaporated to provide **1** as a white solid in almost quantitative recovery yield. *m/z* (ESI, negative) $[M - H]^-$, 470.0409. $C_{20}H_{18}Cl_2NO_8$ requires *m/z* 470.0409; δ_H (400 MHz, CD_3OD) 3.43–3.76 (4H, m, C_2-, C_3-, C_4- and C_5-*H*), 3.90 (2H, s, ArCH_2), 5.53 (1H, d, *J* 6.6, C_1-*H*), 6.35 (1H, dd, *J* 1.0 and 7.9, Ar-*H*), 6.86 (1H, dt, *J* 1.0 and 7.6, Ar-*H*), 7.03 (2H, t, *J* 7.6, Ar-*H*), 7.19 (1H, dd, *J* 1.3 and 7.6, Ar-*H*) and 7.35 (2H, d, *J* 8.2, Ar-*H*); δ_C (100 MHz, CD_3OD) 20.73, 38.60, 72.94, 73.62, 77.17, 77.42, 95.95, 118.79, 122.88, 125.22, 125.86, 129.02, 130.00, 131.48, 132.23, 139.36, 144.53, 172.67 and 175.19.

Mefenamic acid 1-β-*O*-acyl glucuronide 2. The methyl ester **13** (27 mg, 63 μmol) was dissolved in DMSO (32 cm^3) and the solution was poured into 96 cm^3 of 25 mM sodium phosphate buffer (pH 6.0). To the resultant solution, PLE (65 mg, 0.4 mg cm^{-3} incubation mixture) dissolved in an aqueous solution (32 cm^3) was added and incubated at 40 °C for 1.5 h. The conversion yield to **2** was 99% by HPLC analysis; the mobile phase was 50% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM TBAB at a flow rate of 0.7 cm^3 min^{-1} with detection at 283 nm. The reaction mixture was acidified with 6 M HCl (to pH *ca.* 3) and then filtered off. The filtrate was loaded on an Amberlite XAD-4 column (5 g), which was prepared as described above. The column was washed with water (100 cm^3) and then with 50% CH_3CN (50 cm^3) and eluted with 80% CH_3CN (50 cm^3). The fraction was evaporated to give **2** as a white solid in almost quantitative yield. *m/z* (FAB, positive) MNa^+, 440.1312. $C_{21}H_{23}NO_8Na$ requires *m/z* 440.1317; δ_H (270 MHz, CD_3OD) 2.14 (3H, s, ArCH_3), 2.32 (3H, s, ArCH_3), 3.58–3.31 (3H, m, C_2-, C_3- and C_4-*H*), 3.77 (1H, d, *J* 9.6, C_5-*H*), 5.77 (1H, d, *J* 7.6, C_1-*H*), 6.60–6.69 (2H, m, Ar-*H*), 7.03–7.10 (3H, m, Ar-*H*), 7.25 (1H, t, *J* 7.9, Ar-*H*), 8.07 (1H, dd, *J* 1.6 and 8.2, Ar-*H*) and 9.14 (1H, s, N*H*); δ_C (100 MHz, CD_3OD) 14.10, 20.61, 73.47, 73.87, 76.76, 78.01, 95.96, 111.16, 114.45, 117.13, 124.59, 127.16, 128.21, 133.05, 133.76, 135.83, 139.40, 139.69, 151.39, 168.62 and 176.34.

(*S*)-Naproxen 1-β-*O*-acyl glucuronide 3. The methyl ester **14** (38 mg, 90 μmol) was dissolved in DMSO (14 cm^3) and the solution was poured into 56 cm^3 of 25 mM sodium citrate and phosphate buffer (pH 5.5) in which PLE was dissolved (28 mg, 0.4 mg cm^{-3} incubation mixture) and then incubated at 40 °C for 3 h. The conversion yield to **3** was 90% by HPLC analysis; the mobile phase was 45% (v/v) CH_3CN containing 50 mM NH_4OAc (pH 4.5) and 10 mM TBAB at a flow rate of 0.7 cm^3 min^{-1} with detection at 271 nm. The reaction mixture was acidified (to pH *ca.* 3) with 6 M HCl and then filtered off. The filtrate was loaded on an

Amberlite XAD-4 column (5 g), which was prepared as described above. The column was washed with water (100 cm^3) and then with 25% CH_3CN (50 cm^3) and eluted with 80% CH_3CN (50 cm^3). The fraction was evaporated to give **3** as a white solid in almost quantitative recovery yield. *m/z* (SIMS, positive) MH^+, 407.1302. $C_{20}H_{23}NO_9$ requires *m/z* 407.1321; δ_H (400 MHz, CD_3OD) 1.56 (3H, d, *J* 7.2, $CHCH_3$), 3.30–3.50 (3H, m, C_2-, C_3- and C_4-*H*), 3.84 (1H, d, *J* 8.8, C_5-*H*), 3.88 (3H, s, $ArOCH_3$), 3.97 (1H, q, *J* 7.2, $CHCH_3$), 5.53 (1H, d, *J* 8.1, C_1-*H*), 7.09 (1H, dd, *J* 2.4 and 8.9, Ar-*H*), 7.18 (1H, s, Ar-*H*), 7.39 (1H, dd, *J* 1.7 and 8.5, Ar-*H*) and 7.67–7.72 (3H, m, Ar-*H*); δ_C (100 MHz, CD_3OD) 19.23, 46.46, 55.72, 73.02, 73.60, 77.27, 77.60, 95.82, 106.54, 119.88, 127.14, 127.29, 128.26, 130.29, 130.41, 135.27, 136.58, 159.17, 172.87 and 174.92.

Assay of purity of 1-β-*O*-acyl glucuronides 1–3. 1-β-*O*-Acyl glucuronides **1–3** were treated with 0.05 M NaOH at 37 °C for 15 min to complete the hydrolysis of the ester linkage and the amounts of the corresponding carboxylic acids (**5–7**) formed were determined by HPLC. In addition, the same amounts of the glucuronides **1–3** were incubated with β-glucuronidase (from bovine liver, Sigma) in 100 mM AcOH–NaOH buffer (pH 5.0) at 37 °C for 15 min and the amounts of **5–7** formed were also determined. The purities of **1–3** as 1-β-*O*-acyl glucuronides were calculated from the amounts of **5–7** formed under both set of conditions.

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