Syntheses and Characterization of the Acyl Glucuronide and Hydroxy Metabolites of Diclofenac

Jane R. Kenny,[†,‡] James L. Maggs,[†] Xiaoli Meng,[§] Deborah Sinnott,[§] Stephen E. Clarke,[||] B. Kevin Park,[†] and Andrew V. Stachulski*[,§]

Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3GE, United Kingdom, Robert Robinson Laboratories, Department of Chemistry, University of Liverpool, Liverpool L69 7ZD, United Kingdom, and Department of Mechanism and Extrapolation, GlaxoSmithKline, The Frythe, Ware, Hertfordshire AL6 9AR, United Kingdom

Received May 5, 2003

In humans, metabolism of the commonly used nonsteroidal antiinflammatory drug diclofenac **1** yields principally the 4′-hydroxy **2**, 5-hydroxy **3**, and acyl glucuronide **4** metabolites. All three metabolites have been implicated in rare idiosyncratic adverse reactions associated with this widely used drug. Therefore, for mechanistic toxicological studies of **1**, substantial quantities of **2**−**4** are required and their syntheses and characterization are described here. Key steps were a convenient two-step preparation of aniline **5** from phenol, efficient and selective 6-iodination of amide **18**, and high-yielding Ullmann couplings to generate diarylamines **11** and **21**. The acyl glucuronide **4** was obtained by Mitsunobu reaction of **1** (free acid) with allyl glucuronate **23** followed by Pd(0) deprotection, using a modification of a published procedure. We report full characterization of **4** and note that this important metabolite has been made available pure and in quantity for the first time. We report also the metabolic fates of the synthetic metabolites: **2** and **3** were glucuronidated in rats, but only **3** formed glutathione adducts in vivo and by enzymatic synthesis via a quinoneimine intermediate. A previously undescribed glutathione adduct of **3** was obtained by enzymatic synthesis. Compound **4** formed an imine-linked protein conjugate as evinced by sodium cyanoborohydride trapping.

Introduction

Diclofenac **1**[1] (Voltaren) has long been widely used for its antipyretic, analgesic, and antiinflammatory activities. The mechanism of action of **1** is by inhibition of the arachidonic acid cascade at the level of cyclooxygenase-2:[2] the angle of twist between the aryl rings is critical for activity.

In general, **1** has proved to be a highly effective and safe product. Nevertheless, various adverse reactions have been reported, including hepatotoxicity.[3] It has been suggested that the toxicity observed is due at least in part to one or more of the metabolites of **1** and especially the 4′- and 5-hydroxy derivatives, their putative quinoneimine intermediates, and the drug's acyl glucuronide (Figure 1). The acute cytotoxicity of **1** toward isolated rat hepatocytes has been ascribed to the combined actions of the parent compound and its hydroxylated metabolites on mitochondria and to redox cycling between 5-hydroxy and *N*,5-dihydroxy derivatives.[4] It has been postulated that acyl glucuronides may cause immune-mediated adverse drug reactions in man by covalent modification of endogenous proteins.[5] However, direct evidence for the immunogenicity of the acyl glucuronide metabolite is lacking. In humans and rats, the principal metabolites are 4′-hydroxydiclofenac **2**, 5-hydroxydiclofenac **3**, and the acyl glucuronide **4**. Trace amounts of mercapturates of **2** and **3**, thought to be derived from quinoneimine intermediates via glutathione adducts, have been found in rat and human urine.[6] In connection with a program of toxicological evaluations of **1** and its metabolites, we set out to establish robust syntheses of each of these compounds. A number of other groups have studied diclofenac metabolism and its toxicological consequences.[4−12]

The synthesis of **4** has not been reported in detail previously. Both **2** and **3** were described in the definitive structure−activity paper[1a] of analogues of **1** but without details of the preparation of key intermediates. In addition, a confounding conflict between tabular numbers and the numbers in the Experimental Section regarding **3** and its synthetic intermediates is evident in ref 1a. Other preparations of **2** and **3** have been reported[6−8] without experimental detail, and Waterhouse[13] has described a synthesis of deuterated **2**; a preparation of **2** using microbial hydroxylation has also been described.[14] It is vital for both toxicological evaluation and mechanistic studies that the important metabolites **2**−**4** should be made available in quantity and characterized rigorously prior to biological investigation. We now present detailed syntheses of **2**−**4** and summarize their metabolic fates in vivo together with the protein binding behavior of **4**.

Chemistry

4′-Hydroxydiclofenac 2. The key intermediate here is 2,6-dichloro-4-methoxyaniline **5** (Scheme 1). This compound has been made from the expensive 3,5-dichlorophenol in four steps and 12% overall yield[13,15]

* To whom correspondence should be addressed. Tel: 0151 7943542. Fax: 0151 7943588. E-mail: stachuls@liv.ac.uk.
[†] Department of Pharmacology and Therapeutics, University of Liverpool.
[‡] Current address: AstraZeneca R&D Charnwood, Discovery−Physical & Metabolic Science, Bakewell Road, Loughborough, Leics LE11 5RH, U.K.
[§] Department of Chemistry, University of Liverpool.
[||] Department of Mechanism and Extrapolation, GlaxoSmithKline.

10.1021/jm030891w CCC: $27.50
Published on Web 04/27/2004

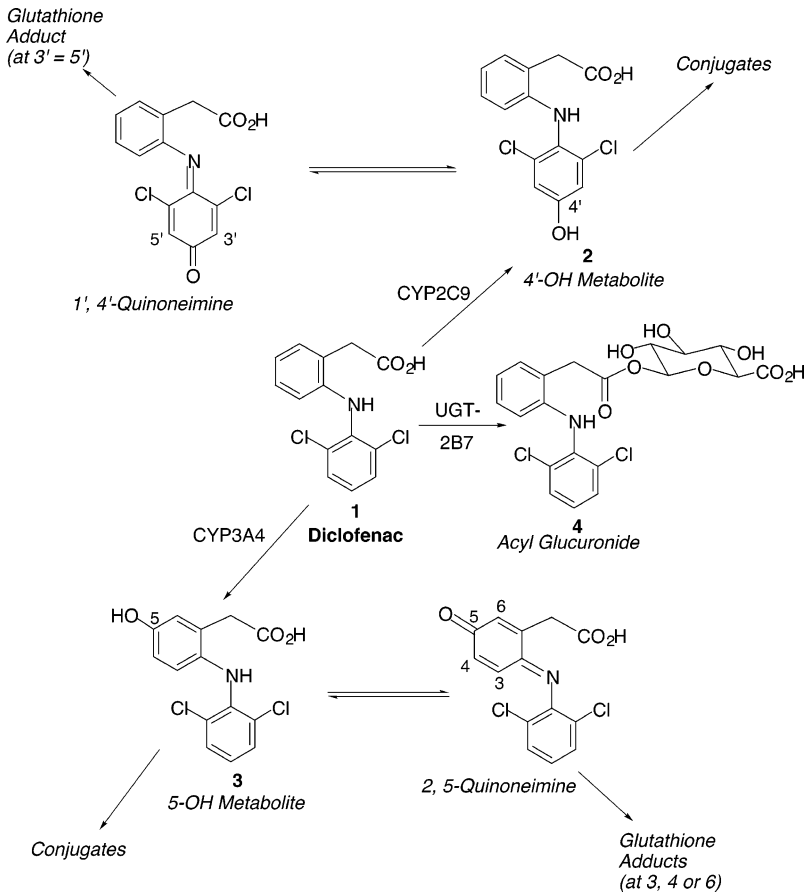
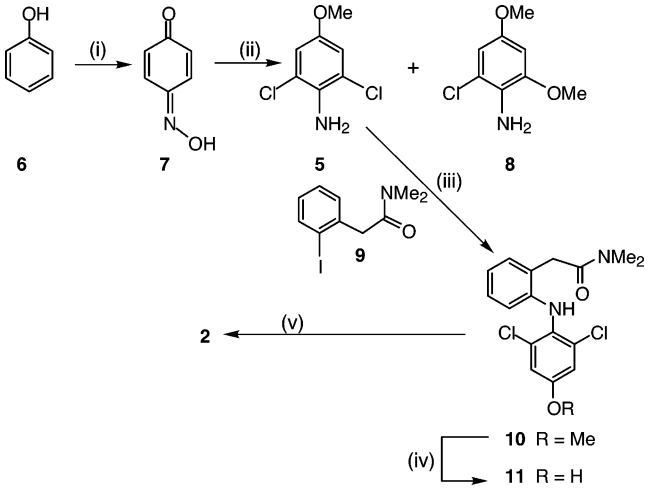


Figure 1. Selected pathways of the in vivo metabolism of **1**. "Conjugates" include glucuronides and sulfate esters but are not all fully characterized. CYP and UGT isozymes are those operative in human liver.

Scheme 1[a]



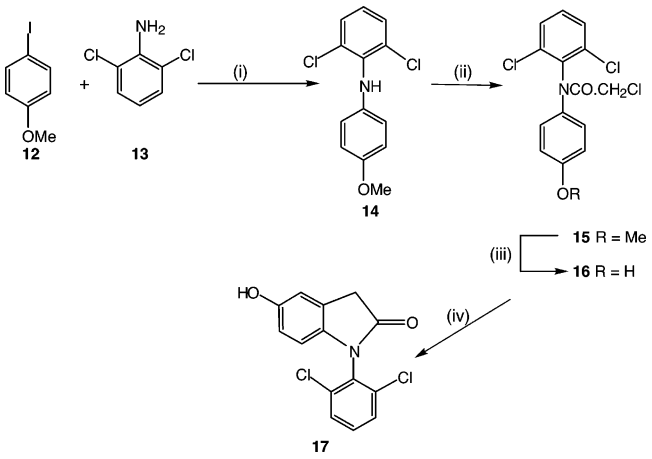
[a] Reagents and conditions: (i) $NaNO_2$, H_2SO_4, 20 °C; (ii) HCl, MeOH−Et_2O, −5 to 0 °C; (iii) **9**, act. Cu, CuI, K_2CO_3, toluene, 110 °C; (iv) BBr_3, DCM, 20 °C; (v) NaOH, aqueous EtOH, 80 °C, N_2.

or by the very low-yielding chlorination of 4-methoxyaniline.[16] We made use of a reported[17,18] two-step synthesis from phenol **6** commencing with nitrosation,[19] which afforded benzoquinone monoxime **7** in 77% yield. Our initial attempts to convert **7** into **5** by reaction with gaseous HCl in dioxan−methanol or methanol−ether afforded mainly 2-chloro-4,6-dimethoxyaniline **8**, noted as a byproduct by Goldschmidt.[18] After considerable experimentation, we found that dropwise addition of a solution of **7** into methanol, through which HCl was passed continuously, and maintaining an external temperature of −5 to 0 °C afforded **5** in quite satisfactory yield (45%) following basification and extraction, now free of **8**.

Standard conversion of 2-iodophenylacetic acid to its *N*,*N*-dimethylamide **9**[20] via the acid chloride (80%) was followed by Ullmann coupling of **5** and **9** using activated Cu powder. This was one of the general methods used by the Ciba-Geigy group[1a] and delivered diarylamine **10** in 82% yield. It proved more expedient to effect *O*-demethylation at this stage; treatment of **10** with BBr_3 proceeded smoothly at 20 °C to give free phenol **11** (81%). Rather surprisingly, this method was reported to fail on a closely related indolone derivative.[13] The final hydrolysis step required very carefully controlled conditions to avoid excessive degradation; eventually, heating an ethanol solution of **11** with 1 M NaOH (8 equiv) at gentle reflux under nitrogen[13] was found to give a satisfactory yield (47%) of **2**. Undoubtedly, the free phenolic group is the source of the problem; base hydrolysis of **10** proceeded smoothly, but BBr_3 treatment of the product gave cyclization to the indolone together with *O*-demethylation. We found later that as in the 5-hydroxy series (vide infra) the use of a Schlenk tube, with rigorous exclusion of oxygen, led to a significant increase in the hydrolysis yield (80%) and a purer product.

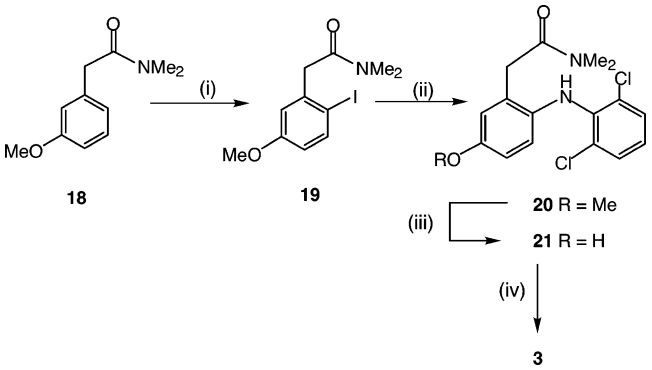
5-Hydroxydiclofenac 3. We initially attempted to prepare this derivative via an intramolecular Friedel−Crafts reaction (Scheme 2), another reaction used in the structure−activity relationship study.[1a] Palladium-

Scheme 2[a]



[a] Reagents and conditions: (i) *rac*-BINAP, $Pd(OAc)_2$, Cs_2CO_3, PhMe, 100 °C, N_2; (ii) $ClCH_2COCl$, 105 °C; (iii) $AlCl_3$, 150 °C; (iv) $AlCl_3$, 200−210 °C.

Scheme 3[a]

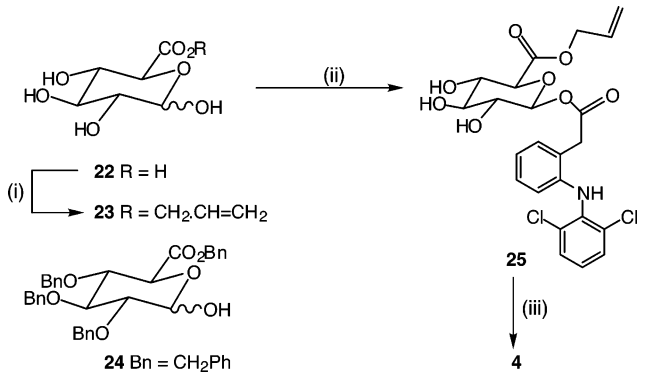


[a] Reagents and conditions: (i) N-Iodosuccinimide, MeCN, reflux; (ii) **13**, act. Cu, CuI, K_2CO_3, toluene, 110 °C; (iii) BBr_3, DCM, 20 °C; (iv) NaOH, aqueous EtOH, 80 °C, N_2.

mediated coupling of 4-iodoanisole **12** and 2,6-dichloroaniline **13** under Buchwald−Hartwig conditions[21,22] afforded a fair (59%) yield of diarylamine **14** after careful chromatography. Following acylation with chloroacetyl chloride, we studied the $AlCl_3$-catalyzed cyclization of the product **15**.[23] At 150 °C, *O*-demethylation without cyclization was observed; after chromatography, the phenol **16** was isolated in low yield (17%) accompanied by much degradation. When **16** was resubmitted to $AlCl_3$ at 200−210 °C, cyclization was observed[23] and the indolone **17** was isolated in 38% yield after chromatography. This could have been hydrolyzed to **3**, but the low yields and extensive chromatography led us to employ a route similar to that employed for **2**.

3-Methoxyphenyl-*N*,*N*-dimethylacetamide **18**[24] (Scheme 3; obtained from the corresponding acid via the acid chloride as described for **9**) was iodinated using a recently described procedure,[25] namely, heating with *N*-iodosuccinimide in acetonitrile at reflux. This method led regiospecifically to the crystalline 6-iodo derivative **19**[26] in very good yield (80% recrystallized). Ullmann coupling of **19** and **13** then gave the crystalline diarylamine **20**, also in very good yield (91%). As in the 4′-OH series, BBr_3-mediated de-*O*-methylation of **20** proceeded smoothly to give **21** (90%). The final hydrolysis proved even more troublesome than for **11**, and for good yields of **3**, it was essential to rigorously exclude

Scheme 4[a]



[a] Reagents and conditions: (i) $CH_2{=}CH{\cdot}CH_2Br$, DBU, DMF, 20 °C; (ii) **1**, Ph_3P, $Pr^iO_2C{\cdot}N{=}N{\cdot}CO_2Pr^i$, THF, DMF, −10 to +20 °C; (iii) $Pd(PPh_3)_4$, pyrrolidine, THF.

oxygen: heating **21** at gentle reflux with 1 M NaOH (8 equiv) in EtOH for 37 h (Schlenk tube) then afforded a 80% yield of **3**. Without this precaution, e.g., simply employing a nitrogen balloon, the best yield of **3** obtained was 19%. The vital importance of excluding oxygen in the hydrolysis steps of the syntheses of **2** and **3** was not previously noted[1a] and the above, we believe, now represent the syntheses of choice for these important metabolites.

Acyl Glucuronide Metabolite 4. Acyl glucuronides are notoriously unstable compounds, being subject to facile hydrolysis and acyl migration at either acidic or basic pH. No one method has yet emerged as a general synthesis of this class of metabolites.[27] We opted for a minimal protection approach via conversion of glucuronic acid **22** to the allyl ester **23**,[28] rather than a fully protected derivative such as **24**, which takes many steps to prepare;[29] the method is given in Scheme 4. This method, i.e., via Mitsunobu coupling,[28] has been previously reported for **4** without synthetic detail.[30]

Under the literature conditions [glucuronic acid, DBU, and allyl bromide in dimethyl formamide (DMF)],[28] we found that the allyl ester **23** produced was contaminated with significant amounts of DBU even after chromatography. After we dissolved this material in CH_2Cl_2 and cooled it, the ester was deposited as a white solid containing only traces (ca. 2%; ^{1}H NMR) of DBU after filtration, and this material, which was very largely the α-anomer by ^{1}H NMR, was successfully progressed. Commercial **1** (Na salt) was converted into the free acid form, and this was coupled to **23** under Mitsunobu conditions.[28] After conventional silica chromatography (giving a mixture of α- and *β*-product anomers in 25% yield), preparative high-performance liquid chromatography (HPLC) afforded the single *β*-anomer **25** as a flaky amorphous solid in 18% yield. The *β*-configuration is confirmed by δ 5.68 (1 H, d, $J = 8.0$ Hz) for the anomeric proton; any traces of the α-anomer (still present after the silica column, removed by preparative HPLC) are distinguishable by δ 6.2 (1 H, d, $J = 3.5$ Hz). Deallylation of **25** using fresh $Pd(PPh_3)_4$ was rapid at 0 °C, but the purification of the product by chromatography was unreliable when methanol was used. Methanol of AR or HPLC grade is often contaminated with trace amounts of amines, which in the presence of methanol leads to catalytic amounts of methoxide and Zemplen deacylation of **4** on the column. Ethanol proved far superior,

being both less nucleophilic per se and free of amine contaminants. In this way, we obtained **4** in 90% yield; no α-/β-scrambling was observed in the deprotection step, as evinced by δ 5.44 (1 H, d, $J = 7.8$ Hz) for the anomeric proton with no trace of α-glucuronide. Thus, **4** is now available in multihundred milligram amounts (we prepared up to 200 mg in one run) for the first time and with authenticated chemical and anomeric purity.

Metabolic Evaluation

Metabolism of 1−4 in the Rat. Following the iv administration of ^{14}C-labeled **1** to bile duct-cannulated rats, **4**, identified by liquid chromatography−mass spectrometry (LC-MS) [*m/z* 470 $[M - 1]^-$; *m/z* 472 $[M + 1]^+$, 296 $[472 - C_6H_8O_6]^+$ (100), 278, 250, 215] and cochromatography with synthetic **4**, was the predominant biliary metabolite (Figure 2). Glucuronides of **2** and **3** [*m/z* 486 $[M - 1]^-$; *m/z* 488 $[M + 1]^+$, 312 $[472 - C_6O_6H_8]^+$ (100), 294, 266, 231] identified by chromatographic comparison with the biliary metabolites obtained when **2** and **3** were administered individually were also major metabolites, although it was not possible to determine directly whether they were ester or ether glucuronides; loss of dehydroglucuronic acid ($C_6O_6H_8$) was characteristic of *O*-glucuronides rather than diagnostic of either ether or ester conjugates. However, the multiplicity of peaks in the *m/z* 486 ion current chromatogram (Figure 2C) when a bile sample was acidified with orthophosphoric acid to only pH 6.5 suggested spontaneous rearrangement (internal acyl migration) of acyl glucuronides.[12] A small amount of a glutathione adduct of a hydroxylated derivative of **1** (*m/z* 617/619, $[M + 1]^+$) was eluted at 14 min, and a lesser amount of an isomer was eluted at 17 min. Using acetonitrile−formic acid as the HPLC eluent, they were shown to cochromatograph with adducts **27** and **28**, respectively (Scheme 5), of the three glutathione conjugates of **3** prepared by enzymatic synthesis. The principal metabolite and **27** yielded similar mass spectra (Table 1). However, because a base peak at *m/z* 342 is obtained from the C-3′ glutathione conjugate of **2** as well as the C-4 conjugate of **3**,[31a] it is possible that this metabolite was a mixture of two regioisomeric thioethers.

Following iv administration of **4**, some of the acyl glucuronide was excreted unchanged in bile, but there was evidence of hydrolysis to aglycone **1** and further metabolism as the glucuronides of **2** and **3** (*m/z* 486/488, $[M + 1]^+$) were present at low levels; direct cytochrome P450-mediated hydroxylation of **4** is unlikely to occur in rats.[30]

Metabolite **2** was excreted in bile as a glucuronide. There was no evidence for a glutathione conjugate. On the other hand, **3** was excreted as a glucuronide [*m/z* 486 $[M - 1]^-$; *m/z* 488 $[M + 1]^+$, 312 $[472 - C_6O_6H_8]^+$ (100), 294, 266, 231] and also as major and minor glutathione conjugates (Figure 3), which cochromatographed with **27** and **28**, respectively; the principal metabolite and **27** yielded similar positive ion mass spectra (Table 1).

Enzymatic Generation of Glutathione Adducts. Using horseradish peroxidase and H_2O_2 to oxidize the hydroxyl derivatives of **1** in the presence of reduced glutathione, **3** yielded glutathione conjugates and unreacted quinoneimine [*m/z* 310/312, $[M + 1]^+$, 292, 264, 231 (100), 201, 194, 166]. Under the same conditions, **2** was not turned over. Unlike **2**, **3** is susceptible to autoxidation in solution.[31] Compound **3** formed all three

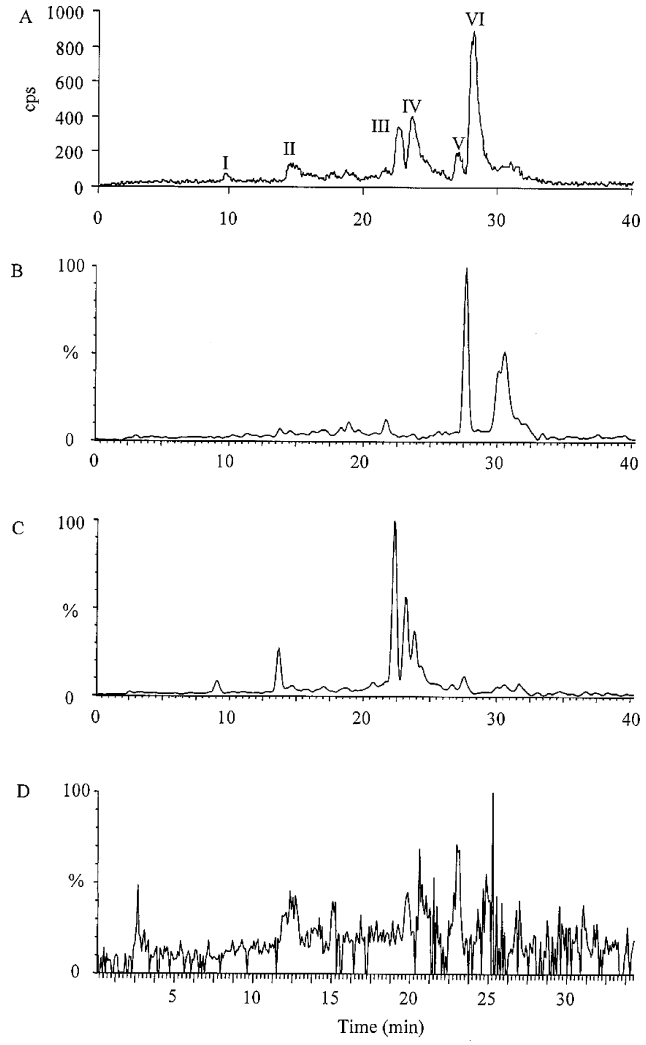
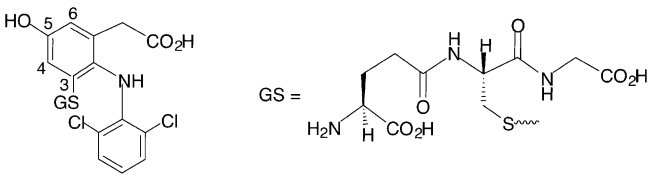


Figure 2. (A) HPLC radiochromatogram of the biliary metabolites of [^{14}C]-labeled **1** (2.12 μmol/kg; 0−1 h collection) in rats where III represents a glucuronide of **2**, IV represents a glucuronide of **3**, and VI represents the glucuronide **4**. (B) Negative ion ES mass chromatogram for *m/z* 470 corresponding to **4**. The maximum absolute signal intensity within this chromatogram was 3.57e3. (C) Negative ion ES mass chromatogram for *m/z* 486 corresponding to glucuronides of **2** and **3**; the multiplicity of *m/z* 486 peaks in bile that was acidified to only pH 6.5 suggests spontaneous rearrangement (internal acyl migration) of acyl glucuronides; the relative areas of the three prominent peaks corresponding to [^{14}C]metabolites III and IV were 1:1.3:0.5. The maximum absolute signal intensity within this chromatogram was 3.31e3. (D) Negative ion ES mass chromatogram for *m/z* 486 in bile collected before administration of [^{14}C]-labeled **1**. Metabolites were resolved with a gradient of acetonitrile in ammonium acetate.

Scheme 5



26 or **27** (Similarly **26** or **27** for 4- and **28** for 6- Glutathione adducts).

Table 1. ES Mass Spectra of *S*-Glutathione Conjugated Derivatives of **1** and **3** Formed by Enzymatic Synthesis and in Vivo[a]

conjugate	mass spectrum
26[b]	*m/z* 617 ([M + 1]$^+$, 27), 542 ([617 − Gly], 18), 488 ([617 − Glu], 41), 385 ([617 − Glu − Cys], 29), 342 ([617 − GS + S], 100), 324 ([342 − H_2O], 11)
27[b]	*m/z* 617 (24), 542 (16), 488 (35), 385 (26), 342 (100), 324 (12)
28[b] (5-OH-6-*S*-glutathionyl-diclofenac)[c]	*m/z* 617 (28), 542 (50), 488 (71), 385 (19), 342 (77), 324 (100)
biliary metabolite of **1**[d]	*m/z* 617 (94), 542 (16), 488 (23), 385 (24), 342 (100), 324 (9)
biliary metabolite of **3**[d]	*m/z* 617 (45), 542 (10), 488 (16), 385 (13), 342 (100), 324 (2)
5-OH-4-*S*-glutathionyl-diclofenac[e]	*m/z* 617 (5), 542 (7), 488 (7), 385 (9), 342 (100), 324 (19)
5-OH-6-*S*-glutathionyl-diclofenac[e]	*m/z* 617 (8), 542 (31), 488 (17), 385 (9), 342 (60), 324 (100)

[a] Analyzed by LC-MS using an acetonitrile−formic acid eluent. [b] Generated from **3** by enzymatic synthesis: horseradish peroxidase/H_2O_2. [c] Identified by comparison with mass spectrum of the synthetic conjugate;[31a] HPLC retention times: **26**, 17.6 min; **27**, 20.9 min; and **28**, 23.6 min. [d] Major component of biliary *m/z* 617/619 mass chromatograms, corresponding chromatographically to enzymatic product **27**. [e] Taken from spectra in ref 31a.

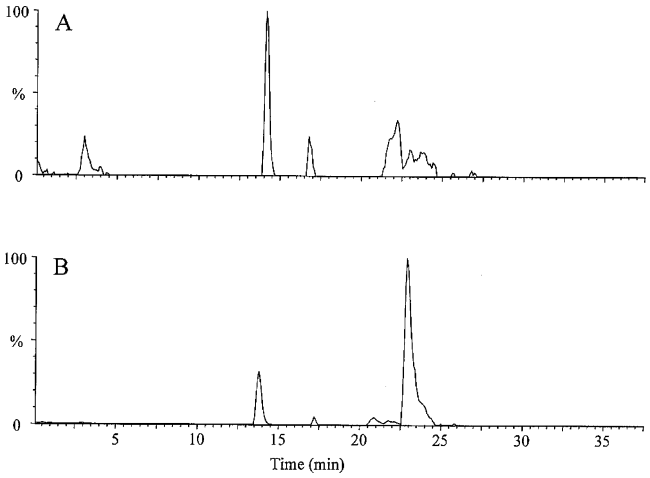


Figure 3. Negative ion ES mass chromatograms for (A) the glutathione conjugates (*m/z* 615/617; 14 and 17 min) and (B) the glucuronide (*m/z* 486/488; 23 min) of **3** (661 μg/kg) eliminated in the bile of rats (0−1 h collection). Metabolites were resolved with a gradient of acetonitrile in ammonium acetate.

of the thioether conjugates (**26**−**28**) that can be derived from its quinoneimine (Figure 1 and Table 1); the proportions of the thioether products, estimated from areas of HPLC-UV chromatographic peaks (λ = 254 nm), were 0.27:1.0:0.84, respectively. Interestingly, only two of the glutathione conjugates derivable from **3** have been prepared chemically and found in the bile of rats administered **1**: in order of elution from a reversed phase HPLC column, 5-OH-4-*S*-glutathionyl- and 5-OH-6-*S*-glutathionyl-diclofenac.[31a] Their electrospray (ES) mass spectra are distinguished by a marked disparity in the ratio of the relative intensities of fragments at *m/z* 324 and *m/z* 342 (0.19 vs 1.7, respectively).[31a] Compound **28** is thereby identifiable as 5-OH-6-*S*-glutathionyl-diclofenac because of its base peak at *m/z* 324 and late elution. The other enzymatic products both yielded mass spectra with relative intensities of *m/z* 324 and *m/z* 342 that were similar to those obtained by Tang et al. from 5-OH-4-*S*-glutathionyl-diclofenac (Table 1). It is proposed that either **26** or **27** is the hitherto unreported 5-OH-3-*S*-glutathionyl-diclofenac conjugate.

Protein Adduct Trapping Experiment. The rearrangement of 1β-acyl glucuronides via internal acyl migration is known to give positional isomers capable of covalently modifying proteins by either transacylation or glycation mechanisms.[32] Acyl glucuronides are commonly observed to be unstable—both in terms of acyl migration and hydrolysis—at physiological pH, while being more stable under acidic conditions.[12] Thus, **4** underwent rearrangement and hydrolysis quite readily at pH 7.4, whereas at pH 5.0 it was essentially stable for several hours (Figure 4). The identities of the glucuronide isomers were confirmed by LC-MS (*m/z* 470 [M − 1]$^-$, 294 [470 − $C_6H_8O_6$]$^-$). Studies on other instances of acyl migration in 1β-acyl glucuronides have shown that the order of isomer elution from reversed phase HPLC columns is 4-, 1α-, 1β-, 3-, and 2-acyl isomer.[12] Upon incubation with human serum albumin, **4**, but neither **1**, **2**, nor **3**, was found to bind covalently to the protein as detected by Western blotting using an anti-diclofenac antibody (Figure 5).[33] The detection of this adduct was dependent on the inclusion of sodium cyanoborohydride. For the stability, and hence detection, of the protein adduct of **4** to be dependent on reduction, it must have been formed via an imine intermediate of the glycation mechanism, which incorporates glucuronic acid in the adduct. The results of earlier work on the isomerization of **4** in aqueous solutions indicate that the glucuronide will have undergone approximately 70% acyl migration during the 2 h incubation, favoring maximum protein binding through glycation.[34,35] However, all of the published mechanistic studies on the formation of covalent protein adducts from diclofenac acyl glucuronide used either microsomally generated conjugate without prior isolation and analysis[35,36] or conjugate isolated from rat bile that could not be obtained in quantities sufficient for verification of composition by ^{1}H NMR.[34] We have now confirmed using a synthetic glucuronide that **4** can react with soluble protein by the same imine mechanism previously found to operate in liver microsomes,[36] when the intermediate was stabilized as an α-aminonitrile by reversible addition of CN^- rather than as an amine by irreversible reduction. The lack of detectable diclofenac haptens on HSA in the absence of coincubated cyanoborohydride contrasts with the finding that 3.8% of [^{14}C]diclofenac acyl glucuronide incubated for 1 h with a 150-fold molar excess of HSA—rather than the 0.048 molar fraction used here—underwent irreversible binding, albeit the adducts were unstable.[34] Neither **2** nor **3** was oxidized by horseradish peroxidase and H_2O_2 to reactive species, which yielded protein adducts detectable with the available anti-diclofenac antibody. However, this antibody was raised against **1** coupled to a carrier protein through an amide linkage;[33] consequently, it might not recognize **1** that is bound via one of its phenyl rings to human serum albumin as a consequence of the protein reacting with a quinoneimine derivative of the drug.

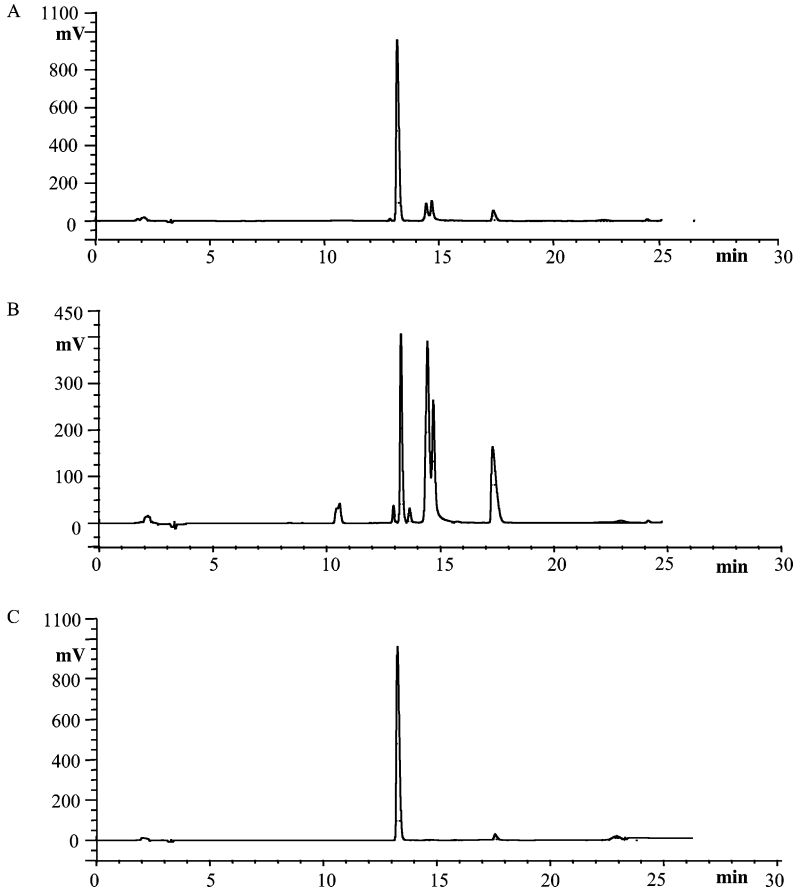


Figure 4. UV−HPLC chromatograms (λ = 254 nm) depicting the pH-dependent rearrangement of **4** in 0.1 M sodium phosphate buffer at 25 °C. (A) pH 7.4 over 1 h, (B) pH 7.4 over 6 h, and (C) pH 5 over 6.5 h. The peak at 13 min is **4**, and that at 17 min is **1** liberated from its acyl glucuronides by spontaneous hydrolysis. Compounds were resolved on a Zorbax 5 μm C-18 column with acetonitrile in ammonium acetate. Products were identified by LC-MS. Horizontal axis, time in min; vertical axis, response in mV.

Summary

We have developed robust syntheses of the 4′- hydroxy **2**, 5-hydroxy **3**, and acyl glucuronide **4** metabolites of **1** suitable for the preparation of several hundred milligrams of each. For what would appear to be the first time, the covalent binding of a haptenating acyl glucuronide (**4**) to protein through a reductively stabilized imine linkage has been demonstrated in vitro using a fully characterized synthetic β-1-*O*-acyl glucuronide of a drug. All three compounds are metabolized in vivo. Again, by use of synthetic material, formation of glutathione conjugates of **3** in rats, presumably through a quinoneimine intermediate, has been confirmed. The full set of three thioether derivatives of this quinoneimine was obtained by peroxidase-catalyzed synthesis. Interestingly, 4′-hydroxydiclofenac **2** under comparable conditions is not oxidized to a quinoneimine. This is consistent with the easier autoxidation of **3** as compared to **2** as expected from the known effect of chlorine substitution on redox potentials of quinonoid substances.[37] The 4′-hydroxy derivative **2** was not metabolized to a thioether conjugate in rats; notwithstanding, it is known to be metabolized to a glutathione conjugate by liver microsomes and is evidently an intermediate of the conjugate's synthesis from **1** in rats and humans;[31] this possibly indicates that the rate of glucuronidation of exogenous **2** by rats is faster than oxidation to the corresponding quinoneimine. The availability of these synthetic metabolites will facilitate further studies of the relationship between the metabolism and the toxicity of diclofenac.

Experimental Section

Chemistry. Ether refers to diethyl ether. Organic solutions were finally washed with saturated brine and dried over anhydrous Na_2SO_4 prior to evaporation under reduced pressure at <40 °C unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed using Merck Kieselgel 60 F 254 silica plates. Preparative column chromatography was performed on Merck 938S silica gel. ^{1}H NMR spectra were recorded on Bruker 250 and 400 MHz instruments, as noted, using the solvents indicated, with tetramethylsilane as the internal standard. Mass spectra were obtained in the electron impact (EI) or chemical ionization (CI) mode using a Fisons Trio 1000 instrument, but using a VG7070E instrument for high-resolution spectra, or in the electrospray (ES) mode using a Micromass LCT mass spectrometer. Standard abbreviations are used for solvents; in addition, DIAD = di-isopropylazodicarboxylate.

4-Benzoquinone Monoxime (6). This compound was prepared according to a literature procedure[19] on a 0.1 M scale; yield, 9.47 g (77%); mp 139−141 °C (lit.[19] mp 137−138 °C). ^{1}H NMR (250 MHz, $CDCl_3$): δ 6.52 (2 H, dd, J = 10.4 and 2.0 Hz, H-2 + H-6), 7.22 (1 H, dd, J = 10.5 and 2.8 Hz, H-5), 7.77 (1 H, dd, J = 10.8 and 2.2 Hz, H-3), and 8.86 (1 H, br s, OH).

2,6-Dichloro-4-methoxyaniline (5). Dry HCl gas was passed into methanol (60 mL), which was stirred at 0 °C until saturation had been achieved. The internal temperature was then lowered to −10 °C (ice−MeOH bath), and a solution of benzoquinone monoxime **6** (5.98 g, 49 mmol) in MeOH (20 mL) and ether (40 mL) was added dropwise with continued HCl passage into the MeOH solution at a rate sufficient to keep

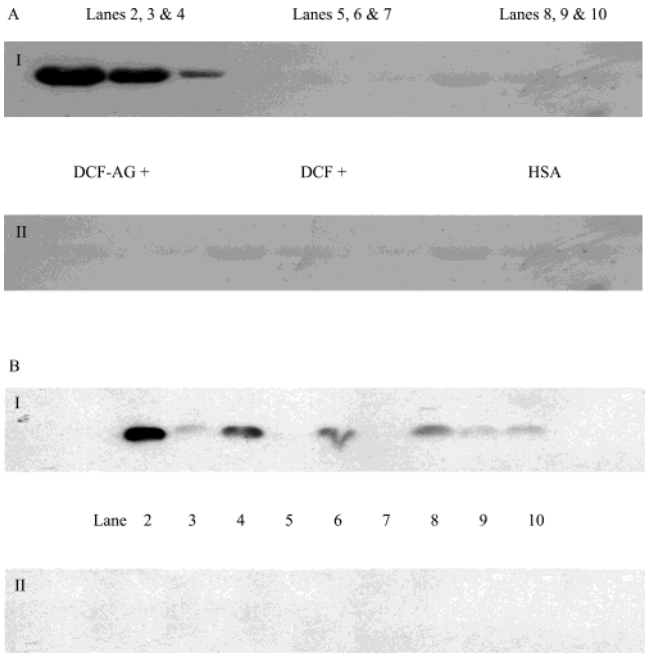


Figure 5. Western blots obtained with anti-diclofenac antibody, showing (A-I) binding of **4** (DCF-AG) to HSA in the presence of sodium cyanoborohydride (20 mM), with 20, 10, and 5 μg of protein in lanes 2, 3, and 4, respectively, and no binding of **1** to HSA (lanes 5−7) or binding of the antibody to HSA (lanes 8−10); (A-II) hapten inhibition by **1** (50 μM) preincubated with antibody of the binding of **4** to HSA; (B-I) effect of sodium cyanoborohydride concentration (20, 10, 2, 1, 0.2, 0.1, and 0.02 mM for lanes 4−10, respectively) on binding of **4** (1.5 mM) to HSA (5 mg/mL), with a positive control for the binding of **4** to HSA in the presence of sodium cyanoborohydride (20 mM) in lane 2 and sodium cyanoborohydride omitted from lane 3; (B-II) hapten inhibition by **1** of the binding shown in B-I.

the temperature below 0 °C. The HCl passage was continued for 1 h after addition was complete, and then, the solid formed was filtered off and washed with ice-cold MeOH. The solid was partitioned between 10% aqueous Na_2CO_3 (50 mL) and ether (2 × 30 mL), and then, the combined organic phases were dried and evaporated to give crude product, which was chromatographed, eluting with 10% EtOAc−hexane, to afford the amine **5** (2.70 g, 29%); mp 69 °C (lit.[18] mp 71 °C). ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.72 (3 H, s, OCH_3), 4.08 (2 H, br s, NH_2), and 6.82 (2 H, s, ArH). Processing of the mother liquors by concentration and then partition between 10% aqueous Na_2CO_3 and ether followed by chromatography as above afforded further product (1.49 g) of equal purity, giving a total of 4.19 g (45%). The byproduct **8** was readily distinguished by ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.72 (3 H, s, CH_3O), 3.77 (2 H, br s, NH_2), 3.82 (3 H, s, CH_3O), 6.36 (1 H, d, J = 2.6 Hz, H-3), and 6.45 (1 H, d, J = 2.6 Hz, H-5).

2-(2-Iodophenyl)-*N*,*N*-dimethylacetamide (9). Oxalyl chloride (0.35 mL) was added dropwise to a solution of 2-iodophenylacetic acid (0.53 g, 1.98 mmol) in anhydrous DCM (12 mL), which was stirred at 20 °C. DMF (1 drop) was added, and the solution was stirred until effervescence ceased (ca. 2 h), then evaporated to dryness, and twice re-evaporated from anhydrous DCM. The product was redissolved in anhydrous DCM (5 mL) and added dropwise to a two-phase system of 40% w/w aqueous dimethylamine (12 mL) in water (35 mL) and DCM (50 mL), which was stirred vigorously at 0 °C. After 1 h, the organic layer was separated, washed with 1 M HCl (25 mL), 10% aqueous Na_2CO_3 (25 mL), and water, and then evaporated to dryness. Purification by chromatography (eluting with EtOAc:hexane, 1:3) afforded the amide **9**[20] as a white semisolid (0.46 g, 80%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.00 and 3.03 (6 H,2 s, 2 × NCH_3), 3.80 (2 H, s, $ArCH_2$), 6.94 (1 H, ddd, J = 7.9, 7.4, and 1.6 Hz, H-5), 7.25 (1 H, dd, J = 7.8 and 1.7 Hz, H-6), 7.30 (1 H, ddd, J = 7.5, 7.2, and 1.6 Hz, H-4), and 7.80 (1 H, dd, J = 7.9 and 0.92 Hz, H-3).

2-[2-(2′,6′-Dichloro-4′-methoxyphenylamino)phenyl]-*N*,*N*-dimethylacetamide (10). A mixture of amide **9** (0.24 g, 0.83 mmol), aniline **5** (0.37 g, 1.90 mmol), anhydrous K_2CO_3 (0.11 g, 0.76 mmol), CuI (0.011 g, 0.052 mmol), and freshly activated Cu powder[38] (0.035 g) was stirred and heated at reflux in anhydrous toluene (5 mL) in a Dean−Stark apparatus filled with 4 Å molecular sieves for 48 h. No amide **9** was then visible by TLC analysis, so the mixture was cooled, filtered, concentrated, and diluted with EtOAc (15 mL), then washed with water, and evaporated. Chromatography, eluting with EtOAc:hexane, 2:3, afforded the acetamide **10** (0.24 g, 82%); mp 161 °C (from EtOAc). Found: C, 57.5; H, 5.2; N, 7.8. $C_{17}H_{18}O_2N_2Cl_2$ requires C, 57.8; H, 5.1; N, 7.9%. ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.98, 3.19 (6 H, 2 s, 2 × NCH_3), 3.80 (3 H, s, OCH_3), 3.84 (2 H, s, $ArCH_2$), 6.36 (1 H, d, J = 8.1 Hz, H-3), 6.82 (1 H, t, J = 7.3 Hz, H-5), 6.94 (2 H, s, H-3′ and H-5′), 7.06 (1 H, t, J = 7.6 Hz, H-4), 7.13 (1 H, d, J = 7.3 Hz, H-6), and 7.46 (1 H, br s, NH). m/z (EI): 353, 355, and 357 (MH^+ for $^{35}Cl_2$, $^{35}Cl + {}^{37}Cl$, and $^{37}Cl_2$, ca. 9:6:1). We later found that a virtually identical yield could be obtained using just 1.5 equiv of aniline **5**.

2-[2-(2′,6′-Dichloro-4′-hydroxyphenylamino)phenyl]-*N*,*N*-dimethylacetamide (11). A 1 M solution of BBr_3 in CH_2Cl_2 (0.5 mL) was added dropwise at 20 °C to a solution of methyl ether **10** (0.050 g, 0.14 mmol) in 1,2-dichloroethane (2 mL). After 1 h, the reaction mixture was added to half-saturated aqueous $NaHCO_3$ (10 mL) and extracted with EtOAc (15 mL); then, the organic phase was separated, washed with water, and evaporated to give phenol **11** as a pale mauve solid (0.039 g, 81%); mp 237−238 °C (from EtOAc−hexane). Found: C, 56.6; H, 4.8; N, 8.2. $C_{17}H_{18}O_2N_2Cl_2$ requires C, 56.7; H, 4.75: N, 8.3%. ^{1}H NMR [250 MHz, $(CD_3)_2CO$]: δ 2.86, 3.20 (6 H, 2 s, 2 × NCH_3), 3.81 (2 H, s, $ArCH_2CO$), 6.17 (1 H, d, ArH), 6.72 (1 h, t, ArH), 6.94 (2 H, s, 3′-H + 5′-H), 6.98 (1 H, m, ArH), 7.15 (1 H, dd, ArH), 7.81 (1 H, br s, NH), and 10.05 (1 H, br s, OH). MS (CI, NH_3): 339, 341, 343 (MH^+ for $^{35}Cl_2$, $^{35}Cl^{37}Cl$, and $^{37}Cl_2$, 100, 67, and 12%).

2-(2′,6′-Dichloro-4′-hydroxyphenylamino)phenylacetic Acid (4′-Hydroxydiclofenac) (2). A solution of amide **11** (0.164 g, 0.48 mmol) in EtOH (5 mL) was heated at reflux under nitrogen with 1 M NaOH (4 mL) for 24 h. The orange solution was cooled, washed with ether (2 × 10 mL), acidified to pH 2 using 3 M HCl, and extracted with EtOAc (25 mL); then, the organic extract was washed with water and evaporated to an orange gum. Purification by partition between ether:EtOAc, 1:1, and half-saturated aqueous $NaHCO_3$ (3 portions), reacidification of the aqueous phase, and re-extraction with ether afforded a light orange solid, which was chromatographed, eluting with a gradient from 25 to 70% EtOAc−hexane; the product was applied to the column in 25% EtOAc−hexane plus a trace of MeOH for solubility. Appropriate fractions were evaporated to yield the product **2** (0.070 g, 47%); mp 173−175 °C (from EtOAc−hexane) (lit.[13] mp 185−189 °C). ^{1}H NMR [400 MHz, $(CD_3)_2SO$]: δ 3.65 (2 H, s, $ArCH_2$), 6.16 (1 H, d, ArH), 6.74 (1 H, approximately t, ArH), 6.82 (1 H, br s, NH), 6.91 (2 H, s, 3′-H + 5′-H), 7.00 (1 H, approximately t, ArH), 7.13 (1 H, d, ArH), and 10.10 (1 H, v br s, OH). MS (EI): 311, 313, 315 (M^+ for $^{35}Cl_2$, $^{35}Cl^{37}Cl$, and $^{37}Cl_2$, 40, 27, and 5%). We found later that performing the reaction in a Schlenk tube with rigorous exclusion of oxygen led to a much improved yield (70%) as in the preparation of **3** from **21**; in this case, the optimum reaction time was 12 h.

2′,6′-(Dichlorophenyl)-4-(methoxyphenyl)amine (14). 4-Iodoanisole **12** (0.38 g, 2.03 mmol), 2,6-dichloroaniline **13** (0.391 g, 2.41 mmol), *rac*-BINAP (0.0105 g, 0.017 mmol), and $Pd(OAc)_2$ (0.0133 g, 0.059 mmol) were added sequentially to redistilled toluene (6 mL), which was stirred in a round-bottom flask previously purged with N_2 for 0.5 h. The resulting bright orange solution was stirred for 10 min, and then, Cs_2CO_3 (0.913 g, 2.80 mmol) was added in one portion and the mixture was again purged with N_2 for 5 min and then heated at 100 °C (external) under N_2 for 20 h. Ether (100 mL) was added,

and the brown reaction mixture was washed with brine, dried over K_2CO_3, and evaporated to a dark green oil. Chromatography, eluting with 5% EtOAc−hexane, afforded the amine **14**[23] as a pale yellow oil (0.32 g, 59%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.77 (3 H, s, CH_3O), 5.77 (1 H, br s, NH), 6.71−6.83 (4 H, 2 d, 2′-, 3′-, 5′- and 6′-Hs), 6.98 (1 H, t, 4-H), and 7.34 (2 H, d, 3- and 5-Hs). MS (EI): 267, 269, and 271 (M^+ for $^{35}Cl_2$, $^{35}Cl^{37}Cl$, and $^{37}Cl_2$, 84, 55, and 10%).

***N*-(Chloroacetyl)-2′,6′-(dichlorophenyl)-4-(methoxyphenyl)amine (15).** Amine **14** (1.32 g, 4.93 mmol) was dissolved in chloroacetyl chloride (11.7 g, 8.25 mL), and the resulting solution was heated at 105 °C for 1.5 h, whereupon TLC indicated complete reaction. Solvent was removed under high vacuum to afford crude amide **15**[23] (2.12 g), which was progressed without purification. ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.79, 3.82 (2 s, 3 H, CH_3O, restricted rotation), 3.97 and 4.17 (2 s, 2H, CH_2Cl, restricted rotation), 6.83−6.93 (2 H, m, ArH), and 7.19−7.57 (5H, m, ArH). MS (CI, NH_3): 344, 346, and 348 (MH^+ for $^{35}Cl_3$, $^{35}Cl_2{}^{37}Cl$, and $^{35}Cl^{37}Cl_2$, 100, 95, and 31%).

***N*-(Chloroacetyl)-2′,6′-(dichlorophenyl)-4-(hydroxyphenyl)amine (16).** An intimate mixture of chloroamide **15** (2.12 g, crude product prepared as above) and finely powdered $AlCl_3$ (2.99 g, 22.4 mmol) was stirred and heated under N_2 at an external temperature of 150−155 °C (oil bath). After 0.5 h, a viscous but sufficiently mobile liquid resulted, and after 1.5 h, reaction appeared complete by TLC; the mixture was cooled and partitioned between EtOAc and 1 M HCl, and then, the aqueous layer was extracted again with EtOAc. The combined organic extracts were evaporated to give a brown oil, which was subjected to chromatography, eluting with 20% EtOAc in hexane, then 30%, to give the product **16** as a brown amorphous solid (0.35 g, 17%). ^{1}H NMR [250 MHz, $(CD_3)_2CO$]: δ 4.05, 4.33 (2 H, 2 s, $ClCH_2$, restricted rotation), 6.80−6.95 (2 H, 2 d, ArH), 7.20 (ca. 0.5 H, d, ArH), 7.40 (ca. 0.5 H, t, ArH), 7.50−7.60 (ca. 3.5 H, m, ArH), 7.67 (ca. 0.5 H, ArH), 8.44, and 8.75 (1 H, 2s, OH). All of the ArH signals are complicated by restricted rotation. MS (CI, NH_3): 330, 332, and 334 (MH^+ for $^{35}Cl_3$, $^{35}Cl_2{}^{37}Cl$, and $^{35}Cl^{37}Cl_2$, 100, 96, and 33%).

1-(2′,6′-Dichlorophenyl)-1,3-dihydro-5-hydroxyindol-2-one (17). The chloramide **16** (0.35 g, 1.06 mmol) was again mixed with $AlCl_3$ (0.5 g, 3.75 mmol) and heated under N_2 in an oil bath maintained at 200−220 °C for 4 h. Workup and chromatographic purification (eluting with 30% EtOAc−hexane) as described for **16** afforded the indolone **17**[23] (0.11 g, 38%) as a brown amorphous solid. ^{1}H NMR [250 MHz, $(CD_3)_2CO$]: δ 3.71 (2 H, s, CH_2Ar), 6.25 (1 H, d, J = 8.3 Hz, 7-H), 6.68−6.71 (1 H, dd, J = 8.3 and 2.5 Hz, 6-H), 6.94 (1 H, d, J = 2.5 Hz, 4-H), 7.57 (1 H, dd, 4′-H), 7.67 (2 H, approximately d, 3′-H + 5′-H), and 8.19 (1 H, s, OH). MS (CI, NH_3): 294, 296, and 298 (MH^+ for $^{35}Cl_2$, $^{35}Cl^{37}Cl$, and $^{37}Cl_2$, 100, 77, and 18%).

(6-Iodo-3-methoxy)phenyl-*N*,*N*-dimethylacetamide (19). 3-Methoxyphenylacetic acid was converted to its *N*,*N*-dimethylamide **18**[24] as described for **9**. A solution of the product (2.20 g, 11.4 mmol) and *N*-iodosuccinimide (5.90 g, 26.27 mmol) in acetonitrile (15 mL) was heated at reflux for 12 h, when the reaction appeared complete by TLC. The final solution was filtered and evaporated, and then, the residue was redissolved in ether (50 mL), washed with 10% aqueous $Na_2S_2O_3$ and water, and evaporated. Recrystallization from EtOAc−hexane gave the iodo compound **19** (2.79 g, 80% in two crops); mp 82−84 °C (lit.[26] mp 86−89 °C). ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.01, 3.03 (6 H, 2 s, 2 × NCH_3), 3.77 (5 H, 2 s, OCH_3 + ArCH_2), 6.56 (1 H, dd, J = 8.7 and 2.9 Hz, 4-H), 6.86 (1 H, d, J = 2.9 Hz, 2-H), and 7.69 (1 H, d, J = 8.7 Hz, 6-H).

2-[(2′,6′-Dichlorophenyl)amino)]-5-methoxyphenyl-*N*,*N*-dimethylacetamide (20). A mixture of iodo compound **19** (0.785 g, 2.46 mmol), 2,6-dichloroaniline **13** (0.759 g, 4.68 mmol), anhydrous K_2CO_3 (0.263 g, 1.86 mmol), CuI (0.026 g), and freshly activated Cu powder[38] (0.086 g) was stirred and heated at reflux in anhydrous toluene (5 mL) for 24 h. Workup as for **10** afforded the product **20** as a crystalline solid (0.79 g, 91%); mp 102 °C (from EtOAc−hexane). Found: C, 57.7; H, 5.2; N, 7.95%. $C_{17}H_{18}Cl_2N_2O_2$ requires C, 57.8; H, 5.1; N, 7.9%. ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.00, 3.19 (6 H, 2 s, 2 × NCH_3), 3.76 (3 H, s, OCH_3), 3.82 (2 H, s, ArCH_2), 6.53 (1 H, d, J = 8.6 Hz, 3-H), 6.67 (1 H, dd, J = 8.6 and 2.9 Hz, 4-H), 6.75 (1 H, d, J = 2.9 Hz, 6-H), 6.90 (1 H, t, 4′-H), 7.17 (1 H, br s, NH), and 7.30 (2 H, d, 3′-H + 5′-H). MS (CI, NH_3): 353, 355, and 357 (M^+ for $^{35}Cl_2$, ^{35}Cl + ^{37}Cl, and $^{37}Cl_2$, 100, 66, and 11%).

2-[(2′,6′-Dichlorophenyl)amino)]-5-hydroxyphenyl-*N*,*N*-dimethylacetamide (21). The methoxyamide **20** was subjected to BBr_3-catalyzed de-*O*-methylation as described for the preparation of **11**. From **20** (0.353 g, 1 mmol) was obtained **21** (0.303 g, 90%); mp 188−191 °C. Found: C, 56.3; H, 4.7; N, 8.2. $C_{16}H_{16}Cl_2N_2O_2$ requires C, 56.65; H, 4.75; N, 8.3%. ^{1}H NMR (250 MHz, CD_3OD): δ 3.03, 3.52 (6 H, 2 s, 2 × NCH_3), 3.87 (2 H, s, ArCH_2), 6.41 (1 H, d, J = 8.6 Hz, 3-H), 6.58 (1 H, dd, J = 8.6 and 2.9 Hz, 4-H), 6.72 (1 H, d, J = 2.9 Hz, 6-H), 7.00 (1 H, approximately t, 4′-H), and 7.38 (2 H, d, 3′-H + 5′-H). MS (CI, NH_3): 339, 341, and 343 (MH^+ for $^{35}Cl_2$, $^{35}Cl^{37}Cl$, and $^{37}Cl_2$, 100, 67, and 12%).

2-[(2′,6′-Dichlorophenyl)amino]-5-hydroxyphenylacetic Acid (3) (5-Hydroxydiclofenac). A solution of 1 M NaOH (2.4 mL) was added to a suspension of amide **21** (0.1 g, 0.29 mmol) in EtOH (1 mL) in a Schlenk tube, and after it was purged with nitrogen, the mixture was heated at 80 °C for 36 h. Workup as described for **2**, but with no chromatography now required, afforded the 5-OH compound **3** (0.071 g, 80%); mp 176−178 °C. Found: C, 53.7; H, 3.5; N, 4.4. $C_{14}H_{11}Cl_2NO_3$ requires C, 53.9; H, 3.55; N, 4.5%. ^{1}H NMR [400 MHz, $(CD_3)_2CO$]: δ 3.80 (2 H, s, ArCH_2), 6.46 (1H, d, J = 8.5 Hz, 3-H), 6.67 (1 H, dd, J = 8.5 and 2.8 Hz, 4-H), 6.75 (1 H, br s, NH), 6.86 (1 H, d, J = 2.8 Hz, 6-H), 7.07 (1 H, t, 4′-H), 7.44 (2 H, d, 3′-H + 5′-H), and 8.04 (1 H, br s, OH). MS (CI, NH_3): 312, 314, and 316 (MH^+ for $^{35}Cl_2$, $^{35}Cl^{37}Cl$, and $^{37}Cl_2$, 100, 67, and 12%).

Allyl α/β-D-Glucopyranuronate (23). This compound, prepared according to the literature procedure,[28] was contaminated with significant quantities of DBU (20 to 30 mol %) following chromatography. Dissolution in DCM followed by cooling to 0 °C afforded a sticky solid, which on filtration, washing with cold DCM, and drying was suitable for progression (DBU content now ca. 2%); the material existed mainly as the α-anomer, although α-/β-ratios were variable with different batches, and the recovery was about 60%. ^{1}H NMR (250 MHz, CD_3OD, α-anomer): δ 3.44 (1 H, dd, J = 9.4 and 3.6 Hz, 2-H), 3.55 (1 H, t, 4-H), 3.72 (1 H, t, 3-H), 4.56 (1 H, d, J = 9.8 Hz, 5-H), 4.71 (2 H, m, $OCH_2CH{=}CH_2$), 5.16 (1 H, d, J = 3.6 Hz, 1-H), 5.26 and 5.39 (2 H, 2 m, $OCH_2CH{=}CH_2$), and 5.96 (1 H, m, $OCH_2CH{=}CH_2$). About 5% of the β-anomer was present. δ (inter alia): 3.21 (0.05 H, dd, J = 9.2 and 7.8 Hz, 2-H), 3.90 (0.05 H, d, J = 9.8 Hz, 5-H), and 4.52 (0.05 H, d, J = 7.8 Hz, 1-H).

Allyl 1-*O*-[2-[(2′,6′-Dichlorophenyl)amino)]phenylacetyl] β-D-Glucopyranuronate (25). To a solution of free acid **1** (301.9 mg, 1.02 mmol) and PPh_3 (270 mg, 1.02 mmol) in THF (4 mL) and DMF (0.5 mL) at −10 °C was added DIAD (200 μL, 1.02 mmol). After 5 min, a solution of allyl glucuronate **23** (120 mg, 0.52 mmol) in THF (1 mL) and DMF (0.25 mL) was added slowly over 10 min. The solution turned dark orange. After 1 h at this temperature and when the reaction was judged to be complete as monitored by TLC, the solvent was removed under vacuum. The product was purified by chromatography on silica gel, eluting with 5−20% EtOH in DCM. Product-rich fractions (UV absorbing and anisaldehyde + ve) were combined and evaporated to give **25** as an α-/β-mixture (2:5; 25%) and purified by preparative HPLC (Phenomenex Semi-Prep Zorbax C-18 250 mm × 10 mm, 2 mL/min, acetonitrile 10−60%, 35 min H_2O, retention time 27 min). This yielded the desired product **25**, solely as the β-anomer, as an amorphous solid (47 mg, 18%). ^{1}H NMR [400 MHz, $(CD_3)_2CO$]: δ 3.52 (1 H, m), 3.61 (1 H, m) and 3.70 (1 H, m, 2′-H, 3′-H and 4′-H), 3.96 (2 H, AB qt, ArCH_2CO), 4.07 (1 H, d, J = 9.5 Hz, 5′-H), 4.65 (2 H, m, $OCH_2CH{=}CH_2$), 5.20 (1 H, dd, J = 10.5 and 1.4 Hz) and 5.36 (1 H, dd, J = 17.2 and 1.6 Hz, $OCH_2CH{=}CH_2$), 5.68 (1 H, d, J = 8.0 Hz, 1′-H), 5.94 (1 H, m, $OCH_2CH{=}CH_2$), 6.49 (1 H, d, J = 8.0 Hz, ArH), 6.78 (1 H, br s, NH), 6.96 (1 H, t, ArH), 7.16 (2 H, m, ArH), 7.31 (1 H, dd,

ArH), and 7.49 (2 H, d, ArH). MS (ES + ve mode): 534 (MNa^+, 100%, $^{35}Cl_2$), 536 (MNa^+, 65%, $^{35}Cl/^{37}Cl$), and 538 (MNa^+, 12%, $^{37}Cl_2$). HRMS [MH^+]: *m/z* calcd for $C_{23}H_{24}{}^{35}Cl^{37}ClNO_8$, 514.0849; found, 514.0844.

1-*O*-[2-[(2′,6′-Dichlorophenyl)amino)]phenylacetyl]-β-D-glucopyranuronic Acid (4) (Diclofenac Acyl Glucuronide). To a solution of allyl ester **25** (0.100 g, 0.24 mmol) in THF (1 mL) at 0 °C was added $Pd(PPh_3)_4$ (0.028 g, 0.024 mmol) followed by pyrrolidine (19 μL, 0.23 mmol). The mixture was stirred for 30 min at this temperature, and when the reaction was judged to be complete as monitored by TLC, the solvent was removed under vacuum. The product was purified by chromatography on silica gel eluting with 20% EtOH−DCM containing 0.1% acetic acid. This yielded the acyl glucuronide of diclofenac **4** as a flaky amorphous solid (90 mg, 98%). ^{1}H NMR [400 MHz, $(CD_3)_2SO$]: δ 3.33 (3 H, m, after D_2O exch., 2′-H + 3′-H + 4′-H), 3.63 (1 H, m, 5′-H), 3.88 (2 H, ABqt, ArCH_2CO), 5.12 and 5.29 (2 H, 2 m, D_2O exch., OHs), 5.44 (1 H, d, J = 7.8 Hz, 1′-H), 6.26 (1 H, d, J = 7.9 Hz, ArH), 6.86 (1 H, t, ArH), 6.97 (1 H, br s, D_2O exch., NH), 7.07 (1 H, m, ArH), 7.21 (2 H, m, ArH), and 7.53 (2 H, d, J = 8.1 Hz, ArH). MS (ES + ve mode): 472 (MH^+, 65%, $^{35}Cl_2$), 474 (MH^+, 40%, $^{35}Cl/^{37}Cl$), and 476 (MH^+, 7%, $^{37}Cl_2$). HRMS [MH^+]: *m/z* calcd for $C_{20}H_{20}{}^{35}Cl_2NO_8$, 472.0566; found, 472.0561.

Metabolism of 1−4 in the Rat. Adult male Wistar rats (250 g body weight, n = 4 per group) were anaesthetized with urethane (1.4 g/mL in isotonic saline; 1.0 mL/kg, ip) and cannulated via the trachea, jugular vein, and the common bile duct. Drug blank bile was collected for approximately 20 min before treatment. [^{14}C]Diclofenac sodium salt **1** (2.12 μmol/kg, 674 μg/kg, 7 μCi/kg), **4** (1 mg/kg), **2** (661 μg/kg), and **3** (661 μg/kg) were dissolved in dimethyl sulfoxide−0.15 M saline (1:4, v/v) immediately before their administration and injected intravenously over 5 min. Bile was collected every hour for 5 h into plastic vials containing 10 μL of orthophosphoric acid (17% v/v; pH 1.8), cooled with ice, and stored at −80°C. It was analyzed by LC-MS. Aliquots of bile (10 μL) were mixed with 4 mL of scintillant for determination of radioactivity.

Enzymatic Generation of Quinoneimines and Glutathione Adducts. Hydroxy derivatives **2** and **3** (1.5 mM) were incubated with horseradish peroxidase (Sigma-Aldrich; 20 units/mL) and H_2O_2 (10 μM) in Hanks balanced salt solution containing reduced glutathione (1 mM) at 37 °C for 2 h. The solutions were analyzed by LC-MS.

Stability of 4 in Aqueous Solution. A solution of **4** (1 mg/mL) in 0.1 M sodium phosphate buffer (pH 5.0 or 7.4) was incubated at 25 °C for 6.5 h. The reaction was monitored periodically by removing aliquots (100 μL) for UV-HPLC analysis.

HPLC and LC-MS Analyses. Samples were eluted from either a Columbus 5 μm C-8 or a Zorbax 5 μm C-18 (for monitoring of **4** in solution) HPLC column (25 cm × 0.46 cm; Phenomenex) with a gradient of acetonitrile (10−50% over 40 min) in 5 mM NH_4OAc, pH 6.9, at 0.9 mL/min. For analysis of glutathione adducts only, a gradient of acetonitrile (15−45% over 30 min; 45−65% over 10 min) in 0.1% formic acid was also used. The eluate was monitored at 254 nm. Split-flow eluate (ca. 50 μL/min) was transferred to the LC-MS interface of a Micromass Quattro II mass spectrometer. The ES source was operated at 80 °C using N_2 as nebulizing and drying gas. Analyte fragmentation was achieved at a cone voltage of 50 V. Radiolabeled metabolites were detected by passing the eluate through a Packard Radiomatic Flow-One βeta A250 counter after mixing with Ultima-Flo AP scintillant (Packard Bioscience BV, Groningen, Netherlands) at 1 mL/min.

Covalent Binding of 1−4 to Human Serum Albumin. The ability of **1**−**4** (1.5 mM) to bind covalently to protein was investigated by incubation with human serum albumin (HSA; Fraction V, Sigma Chemical Co.; 5 mg/mL, 72 μM) in phosphate buffer (0.1 M, pH 7.4) in the presence or absence of sodium cyanoborohydride (0.02−20 mM) for 2 h at 37 °C. Control incubations were HSA in the absence of drug. Additionally, **2** and **3** were incubated with HSA (5 mg/mL) in the presence of horseradish peroxidase (20 units/mL) and H_2O_2 (10 μM) to assess the extent to which quinoneimines might contribute to protein adduct formation. On completion of the incubation, samples were diluted to 4 mg/mL protein and analyzed by Western blotting.

Western Blotting. Protein (5−20 μg protein/well) was electrophoresed in a 10% resolving gel for approximately 45 min,[39] blotted onto a nitrocellulose membrane for 1 h at 70 V and 4 °C, and stained with Ponceau Red to assess transfer. The membrane was immersed overnight in a blocking buffer comprised of 5% powdered milk in 100 mM phosphate buffered saline containing 0.25% Tween-20 (T-PBS) and thereafter washed with T-PBS (1 × 15 min, 2 × 5 min). It was then incubated for 1 h with rabbit anti-diclofenac antibody[33] diluted 1:2000 in blocking buffer. Unbound antibody was removed by washing in T-PBS as before. The membrane was incubated finally for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Amersham Life Technologies). Blots were washed in T-PBS, and immune complexes were visualized using an enhanced chemiluminescence detection system (Amersham Life Technologies). Drug specific antibody recognition was confirmed by hapten inhibition when the anti-diclofenac antibody was preincubated with **1** (50 μM) for 30 min.

Acknowledgment. We are grateful to GSK for a maintenance grant (to J.R.K.), to Elizabeth Bowkett (M.Sc. Liverpool, 2001−2002) and Sylvia Newby for valuable experimental assistance, and to Dr. N. R. Kitteringham for preparation of graphics. The anti-diclofenac antibody was kindly provided by Dr. Urs Boelsterli, Institute of Clinical Pharmacy, University of Basel, Switzerland. The LC-MS system was purchased and maintained by means of grants to B.K.P. from the Wellcome Trust.

References

(1) (a) Moser, P.; Sallmann, A.; Wiesenberg, I. Synthesis and Quantitative Structure−Activity Relationships of Diclofenac Analogues. *J. Med. Chem.* **1990**, *33*, 2358−2368. (b) Sallmann, A. The History of Diclofenac. *Am. J. Med.* **1986**, *80* (Suppl. 4B), 29.

(2) Vane, J. R. Inhibition of Prostaglandin Synthesis as a Mechanism of Action for Aspirin-Like Drugs. *Nature (London), New Biol.* **1971**, *231*, 232.

(3) Boelsterli, U. A.; Zimmerman, H. J.; Kretz-Rommel, A. Idiosyncratic Liver Toxicity of Nonsteroidal Antiinflammatory Drugs: Molecular Mechanisms and Pathology. *Crit. Rev. Toxicol.* **1995**, *25*, 207−235.

(4) Bort, R.; Ponsoda, X.; Jover, R.; Gomez-Lechon, M. J.; Castell, J. V. Diclofenac Toxicity to Hepatocytes: A Role for Drug Metabolism in Cell Toxicity. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 65−72.

(5) Park, B. K.; Pirmohamed, M.; Kitteringham, N. R. Role of Drug Disposition in Drug Hypersensitivity: A Chemical, Molecular, and Clinical Perspective. *Chem. Res. Toxicol.* **1998**, *11*, 969−988.

(6) Poon, G. K.; Chen, Q.; Teffera, Y.; Ngui, J. S.; Griffin, P. R.; Braun, M. P.; Doss, G. A.; Freeden, C.; Stearns, R. A.; Evans, D. C.; Baillie, T. A.; Tang, W. Bioactivation of Diclofenac via Benzoquinone Imine Intermediates. *Drug Metab. Dispos.* **2001**, *29*, 1608−1613.

(7) Bort, R.; Ponsoda, X.; Carrasco, E.; Gomez-Lechon, M. J.; Castell, J. V. Metabolism of Aceclofenac in Humans. *Drug Metab. Dispos.* **1996**, *24*, 834−841.

(8) Bort, R.; Ponsoda, X.; Carrasco, E.; Gomez-Lechon, M. J.; Castell, J. V. Comparative Metabolism of the Nonsteroidal Antiinflammatory Drug, Aceclofenac, in the Rat, Monkey and Human. *Drug Metab. Dispos.* **1996**, *24*, 969−975.

(9) Miyamoto, G.; Zahid, N.; Uetrecht, J. P. Oxidation of Diclofenac to Reactive Intermediates by Neutrophils, Myeloperoxidase, and Hypochlorous Acid. *Chem. Res. Toxicol.* **1997**, *10*, 414−419.

(10) Shen, S. J.; Marchick, M. R.; Davis, M. R.; Doss, G. A.; Pohl, L. R. Metabolic Activation of Diclofenac by Human Cytochrome P450 3A4. Role of 5-Hydroxydiclofenac. *Chem. Res. Toxicol.* **1999**, *12*, 214−222.

(11) Sallustio, B. C.; Holbrook, F. L. In Vivo Perturbation of Rat Hepatocyte Canalicular Membrane Function by Diclofenac. *Drug Metab. Dispos.* **2001**, *29*, 1535−1538.

(12) Akira, K.; Uchijima, T.; Hashimoto, T. Rapid Internal Acyl Migration and Protein Binding of Synthetic Probenecid Glucuronides. *Chem. Res. Toxicol.* **2002**, *15*, 765−772.

(13) Waterhouse, I. Synthesis of Deuterium Labeled 4′-Hydroxydiclofenac. *J. Labelled Compd. Radiopharm.* **1999**, *42*, 1075−1083.
(14) Webster, R.; Pacey, M.; Winchester, T.; Johnson, P.; Jezequel, S. Microbial Oxidative Metabolism of Diclofenac: Production of 4′-Hydroxydiclofenac Using *Epicoccum Nigrum* IMI 354292. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 371−376.
(15) Hodgson, H. H.; Wignall, J. S. Nitrosation of Phenols. Part IV. 3:5-Dichlorophenol. *J. Chem. Soc.* **1927**, 2216−2221.
(16) Holmes, R. R.; Bayer, A. P.; Errede, L. A.; Davis, H. R.; Wiesenfeld, A. W.; Bergman, P. M.; Nicholas, D. L. Studies on Nitroso Compounds. II. Dimerisation of 4-Substituted 2,6-Dichloronitrosobenzenes. *J. Org. Chem.* **1965**, *30*, 3837−3840.
(17) Dargelos, A.; Chaillet, M. Reaction De Fixation De HX Sur Les Systemes Paraquinones-II. *Tetrahedron* **1972**, *28*, 3333−3342.
(18) Goldschmidt, S.; Suchanek, L. Versuche Zur Darstellung 2,6,2′,6′-Tetrasubstituierter Diphenochinone. *Chem. Ber.* **1957**, *90*, 19−28.
(19) Hodnett, E. M.; Prakash, G.; Amirmoazammi, J. Nitrogen Analogues of 1,4-Benzoquinones. Activities Against the Ascitic Sarcome 180 of Mice. *J. Med. Chem.* **1978**, *21*, 11−16.
(20) Oza, V. B.; Smith, C.; Raman, P.; Koepf, E. K.; Lashuel, H. A.; Petrassi, H. M.; Chiang, K. P.; Powers, E. T.; Sachettini, J.; Kelly, J. W. Synthesis, Structure and Activity of Diclofenac Analogues as Transthyretin Amyloid Fibril Formation Inhibitors. *J. Med. Chem.* **2002**, *45*, 321−332.
(21) (a) Wolfe, J. P.; Buchwald, S. L. Improved Functional Group Compatibility in the Palladium-Catalyzed Amination of Aryl Bromides. *Tetrahedron Lett.* **1997**, *38*, 6359−6362. (b) Wolfe, J. P.; Buchwald, S. L. Palladium-Catalysed Amination of Aryl Iodides. *J. Org. Chem.* **1996**, *61*, 1133−1135.
(22) Louie, J.; Driver, M. S.; Hamann, B. C.; Hartwig, J. F. Palladium-Catalyzed Amination of Aryl Triflates and Importance of Triflate Addition Rate. *J. Org. Chem.* **1997**, *62*, 1268−1273.
(23) Graevskaya, I. P.; Azimov, V. A.; Ryabova, S. Y.; Alekseeva, L. M.; Anisimova, O. S.; Syubaev, R. D.; Granik, V. G. Reactions of 2,2,6,6-Tetrachlorocyclohexanone with Substituted Anilines and Ammonia. Synthesis of 5-Nitro and 5-Amino Analogues of Orthophen. *Pharm. Chem. J.* **1998**, *32*, 41−44 (translated).
(24) (a) Yardley, J. P.; Morris Husbands, G. E.; Stack, G.; Butch, J.; Bicksler, J.; Moyer, J. A.; Muth, E. A.; Andree, T.; Fletcher, H., III; James, M. N. G.; Sielecki, A. R. 2-Phenyl-2-(1-hydroxycycloalkyl)ethylamine Derivatives: Synthesis and Antidepressant Activity. *J. Med. Chem.* **1990**, *33*, 2899−2905. (b) Epstein, J.; Plapinger, R. E.; Michel, H. O.; Cable, J. R.; Stephani, R. A.; Hester, R. J.; Billington, C., Jr.; List, G. R. Reactions of Isopropyl Methylphosphonofluoridate with Substituted Phenols. *J. Am. Chem. Soc.* **1964**, *86*, 3075−3084.
(25) Carreno, M. C.; Garcia Ruano, J. L.; Sanz, G.; Toledo, M. A.; Urbano, A. Mild and Regiospecific Nuclear Iodination of Methoxybenzenes and Naphthalenes with N-Iodosuccinimide in Acetonitrile. *Tetrahedron Lett.* **1996**, *37*, 4081−4084.
(26) Carson, J. R.; Almond, H. R.; Brannan, M. D.; Carmosin, R. J.; Flaim, S. F.; Gill, A.; Gleason, M. M.; Keely, S. L.; Ludovici, D. W.; Pitis, P. M.; Rebarchak, M. C.; Villani, F. J. 2-Ethynylbenzenealkanamines. A New Class of Calcium Entry Blockers. *J. Med. Chem.* **1988**, *31*, 630−636.
(27) Stachulski, A. V.; Jenkins, G. N. The Synthesis of *O*-Glucuronides. *Nat. Prod. Rep.* **1997**, *15*, 173−186.
(28) Juteau, H.; Gareau, Y.; Labelle, M. A. Convenient Synthesis of *β*-Acyl Glucuronides. *Tetrahedron Lett.* **1997**, *38*, 1481−1484.
(29) (a) Koto, S.; Miura, T.; Hirooka, M.; Tomaru, A.; Iida, M.; Kanemitsu, M.; Takenaka, K.; Masuzawa, S.; Miyaji, S.; Kuroyanagi, N.; Yagishita, M.; Zen, S.; Yago, K.; Tomonaga, F. Stereoselective Syntheses of α-Glucuronides Using Dehydrative Glycosylation. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 3247−3259. (b) Schmidt, R. R.; Ruecker, E. Stereoselective Glycosidations of Uronic Acids. *Tetrahedron Lett.* **1980**, *21*, 1421−1424.
(30) Kumar, S.; Samuel, K.; Subramanian, R.; Braun, M. P.; Stearns, R. A.; Chiu, S.; Evans, D. C.; Baillie, T. A. Extrapolation of Diclofenac Clearance From In Vitro Microsomal Metabolism Data: Role of Acyl Glucuronidation and Sequential Oxidative Metabolism of the Acyl Glucuronide. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 969−978.
(31) (a) Tang, W.; Stearns, R. A.; Bandiera, S. M.; Zhang, Y.; Raab, C.; Braun, M. P.; Dean, D. C.; Pang, J.; Leung, K. H.; Doss, G. A.; Strauss, J. R.; Kwei, G. Y.; Rushmore, T. H.; Chiu, S.-H. L.; Baillie, T. A. Studies on Cytochrome P-450 Mediated Bioactivation of Diclofenac in Rats and in Human Hepatocytes: Identification of Glutathione Conjugated Metabolites. *Drug Metab. Dispos.* **1999**, *27*, 365−372. (b) Tang, W.; Stearns, R. A.; Wang, R. W.; Chiu, S.-H. L.; Baillie, T. A. Roles of Human Hepatic Cytochrome P450s 2C9 and 3A4 in the Metabolic Activation of Diclofenac. *Chem. Res. Toxicol.* **1999**, *12*, 192−199.
(32) Sallustio, B. C.; Sabordo, L.; Evans, A. M.; Nation, R. L. Hepatic Disposition of Electrophilic Acyl Glucuronide Conjugates. *Curr. Drug Metab.* **2000**, *1*, 163−180.
(33) Kretz-Rommel, A.; Boelsterli, U. A. Selective Protein Adducts to Membrane Proteins in Cultured Rat Hepatocytes Exposed to Diclofenac: Radiochemical and Immunochemical Analysis. *Mol. Pharmacol.* **1994**, *45*, 237−244.
(34) Ebner, T.; Heinzel, G.; Prox, A.; Beschke, K.; Wachsmuth, H. Disposition and Chemical Stability of Telmisartan 1-*O*-Acylglucuronide. *Drug Metab. Dispos.* **1999**, *27*, 1143−1149.
(35) Bolze, S.; Bromet, N.; Gay-Feutry, C.; Massiere F.; Boulieu R.; Hulot, T. Development of an in Vitro Screening Model for the Biosynthesis of Acyl Glucuronide Metabolites and the Assessment of Their Reactivity toward Human Serum Albumin. *Drug Metab. Dispos.* **2002**, *30*, 404−413.
(36) Kretz-Rommel, A.; Boelsterli, U. A. Mechanism of Covalent Adduct Formation of Diclofenac to Rat Hepatic Microsomal Proteins. Retention of the Glucuronic Acid Moiety in the Adduct. *Drug Metab. Dispos.* **1994**, *22*, 956−961.
(37) Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*; Wiley: New York, 1967; Vol. 1, pp 215−219 and refs therein.
(38) Vogel, A. I. *A Textbook of Practical Organic Chemistry*, 3rd ed.; Longman: London, 1956; p 192.
(39) Laemmli, U. K. Cleavage of Structural Protein During the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.

JM030891W