Cyclization of the Acyl Glucuronide Metabolite of a Neutral Endopeptidase Inhibitor to an Electrophilic Glutarimide: Synthesis, Reactivity, and Mechanistic Analysis

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The neutral endopeptidase inhibitor (2R)-2-[(1-{[(5-ethyl-1,3,4-thiadiazol-2-yl)amino]carbonyl}cyclopentyl)-methyl]pentanoic acid **2** is metabolized to acyl glucuronide **3**. Unprecedentedly, at pH 7.4, **3** does not undergo the *O*-acyl migration characteristic of acyl glucuronides but rapid, eliminative cyclization ($t_{1/2}$ at 37 °C, 10.2 min) to glutarimide **4**. Glucuronide **3** was synthesized efficiently via acylation of benzylglucuronate with *N*-benzyloxymethyl-protected **2**. Glucuronide and imide reacted rapidly in aqueous solution, pH 7.4, with amino acids and glutathione to form stable amides and unstable thioesters. Imide **4** acylated eight lysine *N*ϵ-amino groups of human serum albumin. Rapid cyclization of **3** was attributed to attack on the ester linkage by an unusually nucleophilic glutaramide NH (pK_a in **2** = 9.76). *N*-propyl **3** was refractory to acyl migration and cyclization. This suggested a synthetic strategy for preparing analogues of **2** that form chemically stable acyl glucuronides.

Introduction

Recent reports[1,2] from Pfizer scientists described a series of glutaramide analogues **1** (Figure 1) which were potent inhibitors of neutral endopeptidase (NEP[a], EC 3.4.24.11) and therefore of potential value in the treatment of female sexual arousal disorder (FSAD).[3] From this series, the thiadiazole analogue **2**, (2R)-2-[(1-{[(5-ethyl-1,3,4-thiadiazol-2-yl)amino]carbonyl}-cyclopentyl)methyl]pentanoic acid (*R*-**2**[1b]; *R*-**13**[2]), was selected as a development candidate in view of its combined activity profile and pharmacokinetic behavior.

In common with many carboxylic acid-containing drugs,[4] **2** is metabolized initially to its *O*-acyl glucuronide **3**.[2] While this property is shared by the series of structure **1**[1b,2], it was soon realized that **3** had a special reactivity: namely, it cyclized ex vivo at physiological pH to give the glutarimide **4** (Figure 2). This very rapid cyclization was attributed to a highly acidic amide NH and formation of a favorable ring size.[5] Subsequent slow hydrolysis of **4** led to a mixture of **2** and the isomeric acid **5**.

The exceptional nature of this rearrangement and the electrophilic character of imides raises the possibility that *O*-glucuronidation of series **1** compounds in vivo will result in previously unsuspected nonenzymatic pathways, including, especially, unfamiliar modifications to biological macromolecules by the glutarimide. The determinants and characteristics of these reactions are likely to be distinct from those of the well characterized, and to a certain extent predictable,[4,6] transacylation, intramolecular *O*-acyl migration, and consequential protein glycation (imine formation) reactions of conventionally labile acyl glucuronides.[4,7] Potential biological consequences of the latter metabolites' reactivity include the idiosyncratic adverse reactions not infrequently associated with drugs that form acyl glucuronides.[7,8] Unacceptable toxicity has been associated with **2** in some experimental animals.[5] It was therefore a matter of importance to develop an effective synthesis of the acyl glucuronide **3** and to characterize the reactions of **3** and **4** with biological nucleophiles. Despite the very short half-life of **3** and consequent suppression of acyl

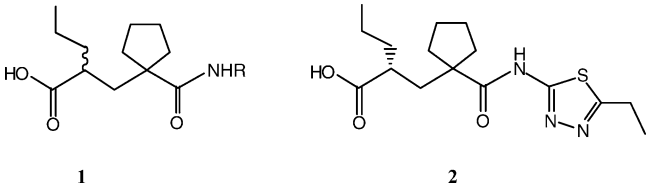

Figure 1. Generic structure of glutaramide NEP inhibitors (**1**) and structure of 1,3,4-thiadiazole analogue **2**.

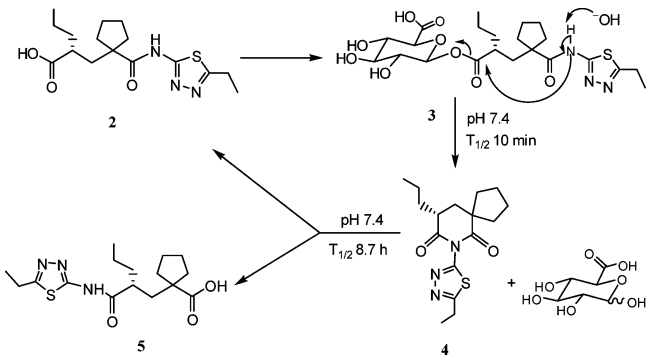

Figure 2. Pathways of formation and rearrangement of acyl glucuronide **3** and hydrolysis of glutarimide intermediate **4**.

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[a] Abbreviations: Bom, benzyloxymethyl; DIAD, diisopropyl azodicarboxylate; FSAD, female sexual arousal disorder; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HSA, human serum albumin; nanoLC-MS/MS, nano liquid chromatography tandem mass spectrometry; NEP, neutral endopeptidase; RSA, rat serum albumin.

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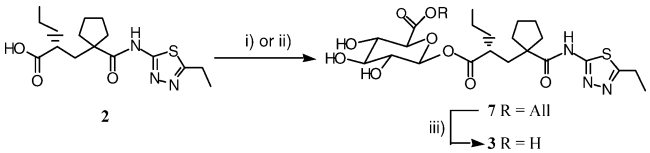
Scheme 1. Direct Synthesis of the 1-β-*O*-Glucuronide from **2**[a]

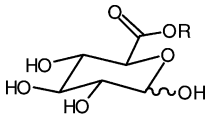


[a] Reagents and conditions: (i) **6**, Ph_3P, DIAD, THF, separate β-anomer by prep HPLC, 30%; (ii) **6**, HATU, NMM, MeCN, ca. 10% **7** + 50% **4**; (iii) $Pd(PPh_3)_4$, pyrrolidine or morpholine, THF, 20 °C, prep HPLC, 70%. All = $CH_2CH{=}CH_2$.

migration, and hence attenuation of the Schiff base-Amadori rearrangement pathway on glycated proteins,[9] it was conceivable that **3** might nevertheless react by rapid acyl transfer to give protein adducts. On the other hand, the cyclization might prove to be a 'safety mechanism' which lessens the risk of covalent protein adduct formation.[4,7,8] The chemical synthesis of such a short-lived and potentially highly reactive metabolite was a challenging task, and its successful achievement is the subject of this report. We report also a definitive confirmation of the rearrangement and hydrolytic pathways shown in Figure 2 using synthetic **3** and **4**, a study of the reactivity of imide **4** with amino acid models of protein nucleophiles, and the covalent modification of serum protein by **4** in vitro.

Results

Chemistry. Methods for acyl glucuronide synthesis have been reviewed.[4,10] It is feasible to prepare acyl glucuronide **3** as an anomeric mixture directly from **2** using the Mitsunobu procedure developed by Juteau et al.[11] Thus condensation of allyl glucuronate **6**[11,12] with **2** under typical Mitsunobu conditions (Ph_3P, DIAD, THF) gave an anomeric mixture (β:α ca. 4:1) of the conjugate **7** in 50% yield along with other products (Scheme 1).



6 R = $CH_2CH{=}CH_2$

13 R = CH_2Ph

Complete separation of the anomers required preparative HPLC, and the desired β-conjugate **7** was eventually isolated in 30% yield. Deprotection of **7** using $Pd(PPh_3)_4$ and pyrrolidine afforded the free acyl glucuronide **3** (70%) without acyl migration or anomerization, again following preparative HPLC. Difficulties were encountered in the purification owing to the extremely short aqueous half-life of **3**. It was best to use 0.5% aq AcOH−acetonitrile, 19:1, as the mobile phase, since **3** is appreciably more stable at mildly acidic pH than at pH 7.4, in common with other acyl glucuronides.[4,13]

We have recently described[12] a new, versatile method of acyl glucuronide synthesis by selective acylation of a glucuronate monoester such as **6**. The great advantage of this procedure is that the conjugate is formed almost entirely (95:5 or better) as the desired β-anomer and preparative HPLC at this stage is unnecessary. Here, however, under our standard conditions (HATU, NMM, **2** + **6** in MeCN), the major product was the imide **4** and the desired conjugate **7** was formed in <10% yield, though entirely as the β-anomer (Scheme 1). Clearly even these mildly basic conditions cannot effectively prevent the cyclization reaction. We therefore studied the use of an appropriate *N*-protecting group to prevent the intramolecular cyclization, followed by a mild final deprotection step.

First we studied *N*-allyl protection, as this should allow a one-step deprotection of both allyl groups when using **6**. Conversion of **2** to the bis-allyl derivative **8** was achieved in excellent yield under basic conditions (2 equiv of allyl bromide, 2 equiv of K_2CO_3, DMF, 20 °C, 80%: Scheme 2). Hydrolysis of the allyl ester was slow but afforded the *N*-allyl acid **9** in 70% yield and high purity. As we anticipated, the *N*-protection completely suppressed the cyclization, and reaction of **9** with **6** (HATU, NMM, MeCN) gave an excellent yield of the protected acyl glucuronide ester **10** (65%).

Deprotection of **10** proved extremely difficult, however. Using $Pd(PPh_3)_4$ and morpholine[14] in THF, the allyl ester, but not the *N*-allyl group, was removed. After much experimentation, we found that **10** was partially deprotected by catalytic transfer hydrogenolysis (Et_3N, HCOOH, Pd−C, 60 °C)[15] but that the *N*-propyl acyl glucuronide **11** was the product. Incidentally this *N*-substituted acyl glucuronide was again highly stable at physiological pH, in contrast to **3**.

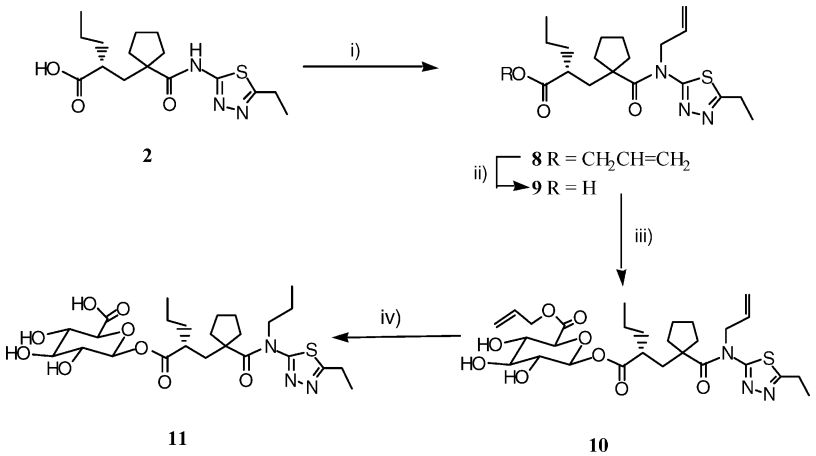
We investigated other protecting groups for the NH moiety of **2**. Introduction of Boc or Z protection was feasible from an ester of **2**, but the resulting acylated amides proved extremely base-labile. When a benzyl ester of **2** was used, the derived *N*-Boc compound could not be hydrogenated without loss of the Boc group, even in EtOAc as solvent.

We therefore resorted to ether protection so that the intermediate would have sufficient base stability. We chose the *N*-benzyloxymethyl (Bom) group[16] which has been successfully used in the synthesis of histidine peptides: deprotection by hydrogenolysis,[16] or relatively mild acidolysis,[17] is then feasible. While the Bom group could be introduced via an ester of **2** by *N*-alkylation (Bom-Cl, Et_3N, CH_2Cl_2) followed by ester hydrolysis, such hydrolyses were sluggish (on the Me or allyl ester), and we devised instead a very efficient three-step, one-pot synthesis from **2** (Scheme 3). Thus silylation of **2** using TBS-Cl and Et_3N (2 equiv each) in CH_2Cl_2 afforded the presumed *N*,*O*-bis-silyl derivative: *N*-silylation was quite slow, requiring 20 h at 20 °C. Reaction of this crude product with Bom-Cl, followed by cleavage of the presumed TBS ester intermediate using TBAF, then afforded the desired *N*-Bom acid **12** in 80% overall yield. Reaction with benzyl glucuronate **13**[18] under similar conditions to the previous (HATU, DABCO, MeCN) afforded the coupled product **14** in 40% yield (unoptimized). While this yield was lower than in some other examples, pure β-product was again obtained: in this case, NMM gave a very sluggish reaction and DABCO was necessary for a reasonable rate (see Supporting Information).

Finally we optimized the deprotection of **14** by hydrogenolysis with 10% Pd−C catalyst. Using Pr^iOH, or Pr^iOH−AcOH mixtures, the benzyl ester was readily removed but the Bom group was only slowly cleaved. However, by using pure AcOH, complete deprotection was achieved in 16 h at 20 °C to deliver highly pure **3**, emphasizing again that the acyl glucuronide is far more stable at mildly acid pH than at physiological pH.[13,19] We did not observe (LC-MS) any *N*-methyl compound which might have been formed via reduction of an iminium intermediate. Final purification was achieved simply by evaporation and trituration of the crude product with acetone to give **3** as a white solid in >95% purity by LC-MS analysis.

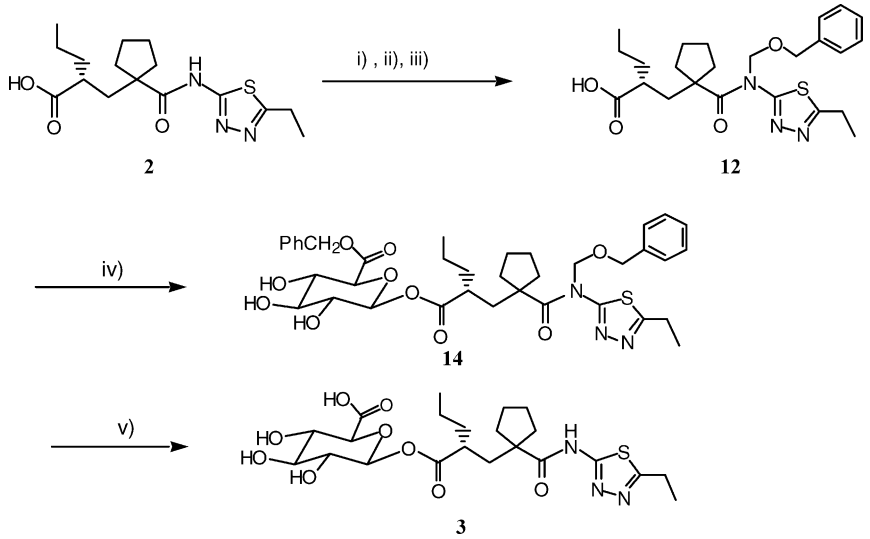
Stability of Acyl Glucuronide 3 and Reactions with Nucleophiles. Synthetic **3** that was free of imide **4** by LC-MS, when incubated in 100 mM sodium phosphate, pH 7.4, at 37 °C underwent the reaction sequence shown in Figure 2.

Scheme 2. Acyl Glucuronide Synthesis Using *N*-Allyl Protection[a]



[a] Reagents and conditions: (i) $CH_2CH{=}CH_2Br$, K_2CO_3 (2 equiv each), DMF, 20 °C, 80%; (ii) NaOH, aq MeOH, 20 °C, 80%; (iii) **6**, HATU, NMM, MeCN, 0−20 °C, 65%; (iv) Et_3N, HCOOH, 10% Pd−C, 60 °C, 70%.

Scheme 3. Synthesis of Acyl Glucuronide **3** Using Benzyloxymethyl (Bom) Protection[a]



[a] Reagent and conditions: (i) TBSCl, CH_2Cl_2, Et_3N, 20 h; (ii) Bom-Cl, Et_3N; (iii) TBAF, THF, 80% for steps i to iii; (iv) **13**, HATU, DABCO, MeCN, 0−20 °C, 40%; (v) H_2-10% Pd−C, AcOH, 80%.

Analysis of serial aliquots by LC-MS (20 to 50 to 70% acetonitrile in 0.1% formic acid, over 30 and 5 min, respectively; required to resolve **3** and **2**) revealed complete degradation of **3** (t_R 18.5 min, *m*/*z* 516 [MH^+], 340 [MH − 176 (dehydroglucuronic acid)]$^+$, 322, 294, 183, 165, 130; *m*/*z* 514 [M − H]$^-$, *m*/*z* 338 [M-176]$^-$, 193 [glucuronic acid − 1]$^-$) after 1 h, yielding **4** (34 min; *m*/*z* 322 [MH^+], 294, 156, 137, 130), **2** (23 min; *m*/*z* 340 [MH^+], 322, 183, 165, 137, 130) and **5** (26 min; *m*/*z* 340, 322, 183, 165, 137, 130) that were identified by chromatographic and mass spectrometric comparisons with synthetic standards. The two carboxylic acids were formed in equal proportions (chromatographic peak areas at 254 nm). The degradation half-life of **3** in phosphate buffer, pH 7.4, at 37 °C was 10.2 min. Cyclization of **3** proceeded slowly in phosphate buffer at pH 6.0 over 6 h, but the glucuronide was stable at pH 3.0. Neither LC-MS analysis of these incubations nor ^{1}H NMR (500 MHz) analysis of incubations performed in deuterated phosphate buffer (pD ca. 7.2) over 10.5 h gave evidence that **3** underwent acyl migration: none of the multiple peaks of *m*/*z* 516 corresponding to positional isomers of **3** was observed,[4,13] and the acyl glucuronide decomposed rapidly in the NMR tube to form only two major products, identified as imide **4** and D-glucuronic acid (Figure 3). In the latter case, decomposition was apparent after only 25 min by the appearance of distinctive doublets attributable to the 1-α, 1-*β*, and 5-α H of D-glucuronic acid. The downfield shift of the pyran ring 2′ C-H signal that is characteristic of acyl group migration from 1*β*-O to 2 α/*β*-O[19] was not seen.

The proposition that rapid cyclization of **3** at neutral pH requires the amide NH to be unusually acidic for nucleophilic attack on the ester linkage[5] was tested by determining the pK_a of **2**. From pH-metric titration, the carboxylate and amide pK_a were 4.74 and 9.76, respectively.

Despite its short half-life in vitro at physiological pH, **3** might directly transacylate nucleophilic amino or thiol species in vivo. This possibility was investigated by incubating **3** with a 40-fold molar excess of either *N*α-acetyl L-lysine **15** or *N*-acetyl L-cysteine **16** in phosphate buffer, pH 7.4, at 37 °C for up to 18 h. Using LC-MS to monitor the reactions, the products corresponded to imide **4** detected as an intermediate, and, respectively, the isomeric amide (**17a**,**b**) and thioester (**18a**,**b**) conjugates of **2** (**17a** and **18a**) and **5** (**17b** and **18b**) that were obtained in similar ratios from **4** itself (Scheme 4; Figure 4; Table 1). The isomeric conjugates produced by reaction of **3** with glutathione were also identical by LC-MS analysis to those derived from **4**. We conclude that the rearrangement of **3** to **4**

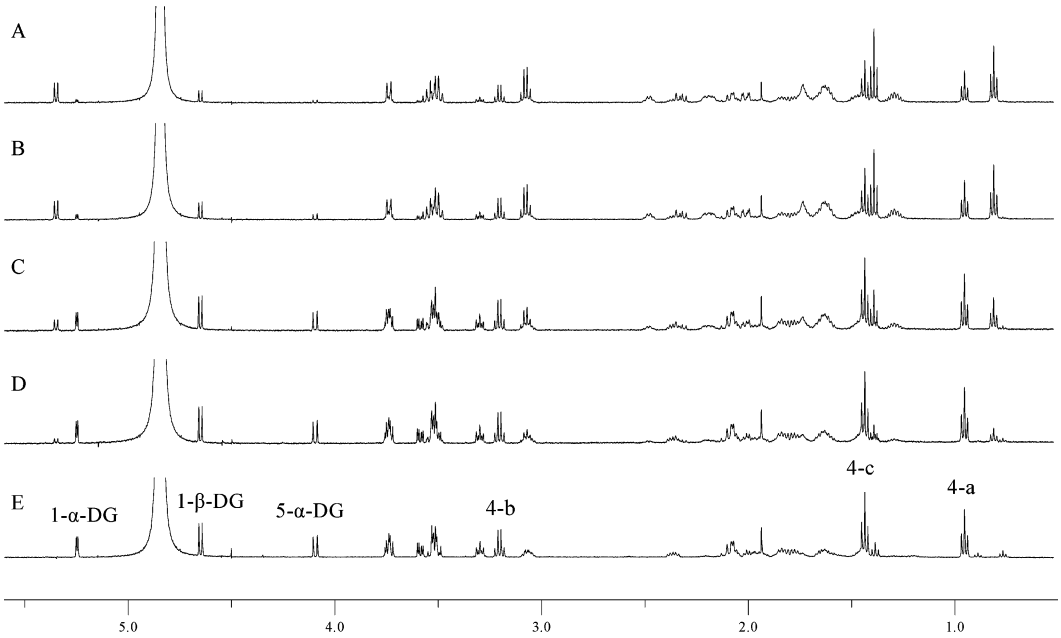
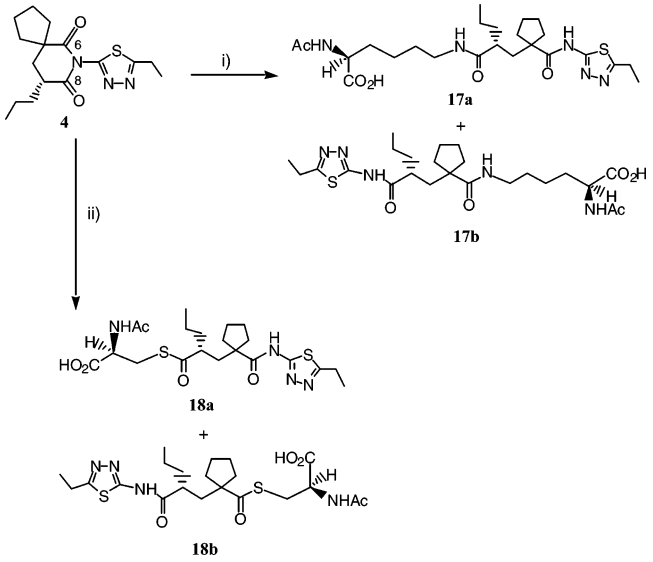


Figure 3. Sequential ^{1}H NMR spectra (500 MHz) of a solution of acyl glucuronide **3** (39 μM) in deuterated phosphate buffer (pD ca. 7.2; 20 °C) showing degradation of **3** to imide **4** (see Figure 2) and D-glucuronic acid (**DG**). Incubation times: A = 25 min, B = 42 min, C = 104 min, D = 160 min, E = 10 h 30 min. 1-β H of acyl glucuronide **3** (δ 5.35, 1 H, d). **DG**: 1-α H, 1-β H and 5-α H (1 H, d) of D-glucuronic acid. **4-a** (3H, t), **4-b** (2H, q), and **4-c** (3H, t) correspond to the CH_3 of the propyl group, the CH_2 of the ethyl group, and the CH_3 of the ethyl group of **4**, respectively.

Scheme 4. Reaction of Imide **4** with Amino Acid Nucleophiles[a]



[a] Reagents and conditions: (i) *N*α-acetyl L-lysine **15**, EtOH, $Na_2B_4O_7$/NaCl buffer, pH 9.22 ± 0.02, (ii) *N*-acetyl L-cysteine **16**, Et_3N, DMAP, acetonitrile. **17a** and **18a** were the major products under these conditions.

is sufficiently fast that the observed products of nucleophilic attack may be attributed to the intermediacy of **4**.

Stability of Imide 4 and Reactions with Nucleophiles. Imide **4** is readily available by treatment of acid **2** with DIC and HOBt in the presence of *N*-methylmorpholine. In order to rationalize an earlier observation that isomeric acid **5** is a major metabolite of **2** in vivo,[20] we first showed that opening of **4** in aqueous conditions at pH 7.4 yielded a mixture of **2** and **5** (Figure 2), identified by comparisons with synthetic standards, in a roughly 1:1 ratio. Carboxylic acid **2** did not cyclize spontaneously. The degradation half-life of **4** in phosphate buffer, pH 7.4, at 37 °C was 8.7 h, but the imide was resistant to hydrolysis at pH 6.0. When synthetic **4** was hydrolyzed under these conditions over 4.5 h, the ratio of **2**:**5** (0.83−0.86, n = 5, between 60 min and 4.5 h) showed a slight though persistent bias in favor of **5** except at 15 min, when the ratio was 1.0. The ratio was lower (0.65) when **4** (500 μM) was incubated with human serum albumin (HSA; 15 μM) under the same conditions but slightly higher (0.9) when it was incubated with rat serum albumin (RSA). These effects might be attributable to protein-mediated hydrolysis: in addition to its more familiar esterase activity,[21] HSA is known to possess β-lactamase activity[22] and aryl acylamidase activity which accounts for approximately 10% of that activity in plasma.[23]

Imide **4** was reacted with model nucleophiles *N*α-acetyl L-lysine **15** and *N*-acetyl L-cysteine **16** (Scheme 4). The reaction of **4** with **15** occurs satisfactorily in aqueous ethanol at pH 9, where **15** exists largely in its free base form and can overcome the background hydrolysis of **4**; a mixture of amides **17a** and **17b** was obtained in a 3:1 ratio determined by HPLC analysis with UV detection. At pH 7 only **2** and **5** resulted. The isomers were separable by preparative HPLC and showed sufficient differences in their ^{1}H NMR for assignment to amides of **2** and **5**, respectively. Reaction of **4** with **16** was effected in acetonitrile under base catalysis and similarly a mixture of isomeric thioesters **18a** and **18b** (2.9:1 ratio by HPLC) resulted, separable by chromatography. These thioesters were relatively unstable on standing or in aqueous solution but were convincingly characterized. It was deduced from preferential formation of **17a** and **18a** that nucleophilic attack on the imide by **15** and **16**, unlike hydrolysis, occurred selectively at the apparently less hindered C-8 carbonyl. In phosphate buffer, pH 7.4, at 37 °C rearrangement of purified **18a** to **4** proceeded to completion within 1 h and was extensive even at pH 6.0. However, the thioester was stable at pH 3.0 for a least 5 h. Incubation of **18a** at pH 7.4 and 37 °C with a 40-fold molar excess of glutathione for 6 h yielded small amounts of two isomeric glutathione conjugates. The *N*-acetyl L-cysteine conjugate was much more stable in the presence of glutathione, but it was not possible to attribute the glutathione conjugates' formation to transacylation of **18a** because of the formation of a small amount of **4**.

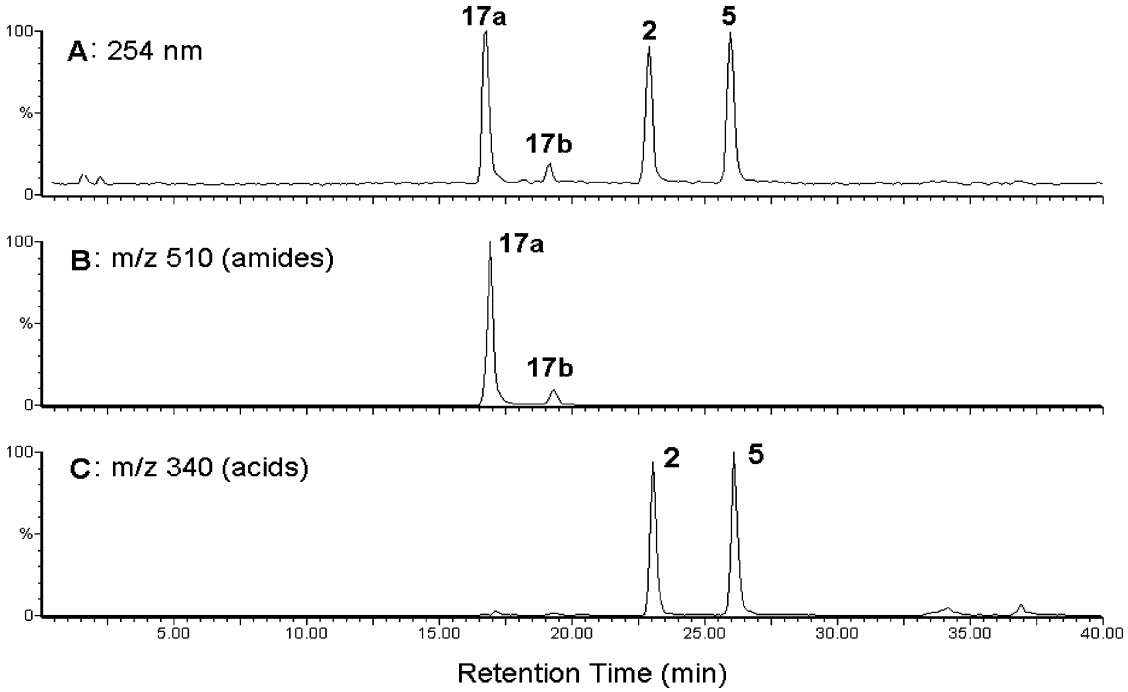


Figure 4. Chromatographic analysis (reversed-phase HPLC) of the products formed during an 18-h incubation of acyl glucuronide **3** with *N*α-acetyl L-lysine **15** (40-fold molar excess). (A) UV chromatogram; (B) mass chromatogram ([MH$^+$]) for amides **17a** and **17b**; (C) mass chromatogram ([MH$^+$]) for acids **2** and **5** formed by hydrolysis of intermediate imide **4** (produced by cyclization of **3**).

Table 1. Conjugates Formed by the Reaction of Imide **4** with Amines and Thiols in Aqueous Solution[a]

nucleophile	% of products[b]	isomeric conjugates[c]	conjugate stability[d]
cysteine	100	2 (1:2.6)	stable
*N*α-Ac-cysteine (**16**)	100	2 (1:1.6)[e]	unstable
glutathione	80	2 (1:12.3)	unstable
lysine	47	4 (2.3:9.5:1:1.3)	stable
*N*α-Ac-lysine (**15**)	52	2 (9.7:1)[e]	stable
*N*α-Ac-lysine Me ester	58	2 (17.5:1)	stable
glycine	53	2 (8.9:1)	stable
arginine	21	2 (3.4:1)	stable
serine[f]	18	2 (2.2:1)	stable

[a] Imide **4** (3 mM) was incubated with nucleophile (120 mM) in phosphate buffer, pH 7.4, at 37 °C for 1 h. Incubations were analyzed by LC-MS (MeCN−NH_4OAc except for *N*α-Ac-lysine and *N*α-Ac-lysine Me ester, which required MeCN−formic acid). [b] After 1-h incubation; estimated from integration of UV (254 nm) chromatogram peaks of the conjugates and isomeric carboxylic acids **2** and **5** (Figure 2), identified by LC-MS. [c] After 1-h incubation. The proportions of the isomeric conjugates were estimated from integration of selected mass chromatogram peaks (*m*/*z* for [MH$^+$]); conjugates are given in order of elution from a reversed-phase column. [d] Unstable conjugates were those that hydrolyzed extensively to carboxylic acids **2** and **5** during the 15−21 h after analysis at 1 h. [e] Identification of conjugates of **15** and **16** confirmed by comparison with standards **17a**,**b** and **18a**,**b**, respectively (Scheme 4). The major amide under these conditions was **17a** (amide of **2**); thioethers **18a** and **18b** were formed in approximately equal amounts. [f] Two additional stable conjugates, less polar than those found after 1 h, obtained after 20 h. Ratio of isomers at 20 h, 2.6:1:2.3:3.1.

Although amide conjugates had not been obtained from **4** and a 2-fold molar excess of *N*α-acetyl L-lysine **15** in aqueous ethanol at pH 7, isomeric conjugates of **2** and **5** were generally produced in good yield when the imide was incubated at pH 7.4 and 37 °C with a 40-fold excess of **15** (**17a** (major) and **17b** (minor); Figure 4), *N*α-acetyl L-lysine methyl ester, L-arginine, and L-glycine (Tables 1 and 2). L-Lysine was monoacylated at both the *N*α- and *N*ε-amine groups. No diacylated L-lysine was found. *N*α-acetyl L-arginine was unreactive. The resolution of two and four conjugates of L-serine after 1 and 20 h, respectively, taken with the observation that *N*α-acetyl L-serine gave no trace of product at 1 and 20 h, suggested that stable esters of **2** and **5** are formed more slowly than *N*α-amides. Two isomeric conjugates of L-histidine (ratio, 3:1), seen in trace amounts at 1 h, represented 8% of imide turnover after 20 h. However, the positions(s) of substitution could not be determined. No derivatives of L-proline were found. Pairs of conjugates were also obtained from *N*-acetyl cysteine **16** (**18a** and **18b**) and glutathione, but the thioesters were unstable under the above conditions and degraded to acids **2** and **5**. This may have occurred via **4**, which, as noted above, is created spontaneously from **18b**. The susceptibility of thioesters of **16** to hydrolysis at pH 7.4 has been observed previously.[24] Cysteine and **16** appeared to compete with hydrolysis of **4** more effectively than the other nucleophiles: after 1 h, turnover of **4** in incubations containing cysteine or **16** was only attributable to conjugate formation (Table 1). Although the formation of two stable conjugates from L-cysteine over 1 h implies selective reaction at the amine function, the absence of thioester products might have been due in part to initial S-acylation followed by rapid S to N migration as reported for thioesters of γ-lactones[25] and hexahydrophthalic anhydride.[24] Glutathione disulfide was unreactive.

Site-Selective Formation of Amide Adducts between Imide 4 and Proteins. HSA and RSA (15 μM) incubated with lower concentrations of **4** (5 and 50 μM) did not yield sufficient quantities of modified tryptic peptides for adequate characterization by nano liquid chromatography tandem mass spectrometry (nanoLC-MS/MS). At the end of a 16-h incubation of the highest concentration of **4** (500 μM) with either HSA or RSA (15 μM), all but minute traces of imide, detectable by LC-MS but not by UV absorption, had been hydrolyzed to carboxylic acids **5** and **2** (1.5:1.0 and 1.1:1.0, respectively). The masses of hypothetical HSA and RSA tryptic peptides modified by addition of **4** (321 amu) were calculated from the amino acid sequences of these proteins in the Swiss-Prot database (entries P02768 and P02770, respectively) minus the first 24 *N*-terminal residues not found in the circulating proteins. First, it was established that peptides

Table 2. Analytical Characteristics of Conjugates Formed by the Reaction of Imide **4** with Amines and Thiols[a]

nucleophile	t_R conjugates (min)[b]	mass spectrum[c]
cysteine	27.6, 32.6	*m/z* 443 ([MH$^+$]), 322([MH − cysteine]$^+$), 314 ([MH − AET]$^+$), 296, 286, 280, 268, 262, 165, 130 ([AET+H]$^+$)
*N*α-Ac-cysteine (**16**)	31.9, 34.8[d]	*m/z* 485 ([MH$^+$]), 356 ([MH − AET]$^+$), 322, 146, 130
glutathione	26.5, 29.2	*m/z* 629 ([MH$^+$]), 500 ([MH − AET]$^+$), 322 ([MH − GSH]$^+$), 130
lysine	21.6, 25.2, 26.2, 29.1	*m/z* 468 ([MH$^+$]), 339 ([MH − AET]$^+$), 322, 321, 293, 183, 165, 130
*N*α-Ac-lysine (**15**)	16.6, 19.0[d]	*m/z* 510 ([MH$^+$]), 381 ([MH − AET]$^+$), 363, 335, 322, 293, 189, 126
*N*α-Ac-lysine Me ester	19.3, 21.7	*m/z* 524 ([MH$^+$]), 395 ([MH − AET]$^+$), 363, 335, 322, 203
glycine	24.8, 30.1	*m/z* 397 ([MH$^+$]), 322, 268 ([MH − AET]$^+$), 250, 240, 222, 194, 165,130
arginine	23.1, 27.7	*m/z* 496 ([MH$^+$]), 367 ([MH − AET]$^+$), 322, 308, 210, 175, 130
serine[e]	23.1, 28.3, 29.5, 31.6	*m/z* 427 ([MH$^+$]), 322, 298 ([MH − AET]$^+$), 280, 130
histidine	22.1, 27.2	*m/z* 477 ([MH$^+$]), 348 ([MH − AET]$^+$), 330, 322, 156

[a] Imide **4** (3 mM) was incubated with nucleophile (120 mM) in sodium phosphate buffer, pH 7.4, at 37 °C for 1−22 h. [b] Retention times on C-8 column (MeCN−NH_4OAc except for *N*α-Ac-lysine and *N*α-Ac-lysine Me ester, which required MeCN−formic acid gradient). [c] Electropspray MS. AET = 2-amino-5-ethyl-1,3,4-thiadiazole moiety (129 amu). Corresponding mass spectra of isomeric carboxylic acids **2** and **5**: *m/z* 340 ([MH$^+$]), 322, 294, 211 ([MH − AET]$^+$), 183, 165, 137, 130; and **4**: *m/z* 322 ([MH$^+$]), 294, 165, 156, 137, 130. [d] Conjugates of **15** were **17a** (16.6 min; amide of **2**) and **17b**; conjugates of **16** were **18a** (34.8 min; thioester of **2**) and **18b** (Scheme 4). [e] The two less polar serine conjugates were seen after 20 h but not after 1 h.

Table 3. Tryptic Peptides of Modified HSA Identified as the Sites Acylated in Vitro by Imide **4**[a]

modified peptide[b]	rt (min)[c]	*m/z* (predicted)[d]	*m/z* (found)[d]	residues	sequence[e]	modified lysine
MT1(H)	45.2	688.9	688.8	137−144	**K**(+)YLYEIAR	K-137
MT2(H)	26.3	534.8	535.0	191−197	ASSA**K**(+)QR	K-195
MT3(H)	37.5	634.8	635.1	198−205	L**K**(+)C(Cm)ASLQK	K-199
MT4(H)	48.2	670.9	671.1	210−218	AF**K**(+)AWAVAR	K-212
MT5(H)	42.1	981.0	981.4	414−428	**K**(+)VPQVSTPTLVEVSR	K-414
MT6(H)	32.1	580.2	580.5	433−439	VGS**K**(+)C(Cm)C(Cm)K	K-436
MT7(H)	45.6	725.5	725.7	525−534	**K**(+)QTALVELVK	K-525
MT8(H)	33.1	569.8	570.0	539−545	AT**K**(+)EQLK	K-541

[a] HSA (15 μM) was incubated with imide **4** (500 μM) in phosphate buffer, pH 7.4, at 37 °C for 16 h. Modified protein was reduced, alkylated,and hydrolyzed with trypsin. Modified peptides were characterized by nanoLC-MS/MS. [b] MT(H) = tryptic peptides of HSA modified by addition of **4**. [c] Retention times of modified peptides on a PepMap 3-μm C-18 Nano Series column. [d] *m/z* for $[M + 2]^{2+}$ of the modified peptide arising from merging of two theoretical tryptic peptides due to missed cleavage adjacent to the acylated lysine. [e] C(Cm) = carboxyamidomethylated cysteine residue. **K** = proposed site of acylation by imide **4**. (+) = site of missed tryptic cleavage. Amino acid sequences are derived from the entry for HSA (ALBU-HUMAN; P02768) in Swiss-Prot minus the first 24 *N*-terminal residues not found in the circulating protein. Residues are numbered for this sequence of 585 amino acids.

Table 4. Tryptic Peptides of Modified RSA Identified as the Sites Acylated in Vitro by Imide **4**[a]

modified peptide[b]	rt (min)	*m/z* (predicted)[d]	*m/z* (found)[d]	residues	sequence[e]	modified lysine
MT1(R)	43.5	639.4	639.4	187−195	E**K**(+)ALVAAVR	K-188
MT2(R)	33.5	674.8	674.8	198−205	M**K**(+)C(Cm)SSMQR	K-199
MT3(R)	38.2	890.9	891.0	275−286	LQAC(Cm)C(Cm)D**K**(+)PVLQK	K-281
MT4(R)	39.9	761.1	761.1	411−428	YTQ**K**(+)APQVSTPTLVEAAR	K-414
MT5(R)	35.7	920.9	921.0	433−445	VGT**K**(+)C(Cm)C(Cm)TLPEAQR	K-436
MT6(R)	46.4	711.4	711.4	525−534	**K**(+)QTALAELVK	K-525
MT7(R)	60.7	822.1	822.1	539−557	ATEDQL**K**(+)TVMGDFAQFVDK	K-545

[a] RSA (15 μM) was incubated with imide **4** (500 μM) in phosphate buffer, pH 7.4, at 37 °C for 16 h. Modified protein was reduced, alkylated, and hydrolyzed with trypsin. Modified peptides were characterized by nanoLC-MS/MS. [b] MT(R) = tryptic peptides of RSA modified by addition of **4**. [c] Retention times of modified peptides on a PepMap 3-μm C-18 Nano Series column. [d] *m/z* for $[M + 2]^{2+}$ of the modified peptide arising from merging of two theoretical tryptic peptides due to missed cleavage adjacent to the acylated lysine. [e] C(Cm) = carboxyamidomethylated cysteine residue. K = proposed site of acylation by imide **4**. (+) = site of missed tryptic cleavage. Amino acid sequences are derived from the entry for RSA (ALBU-RAT; P02770) in Swiss-Prot minus the first 24 *N*-terminal residues not found in the circulating protein. Residues are numbered for this sequence of 584 amino acids.

containing the only cysteine residue of HSA and RSA not forming a disulfide bridge (cysteine-34) had not been modified by **4**. When attempting to predict the sites of proteolysis of *N*-acylated proteins, and therefore the sequences of modified peptides, it was assumed, as confirmed by numerous studies on HSA modified by acylation or alkylation at many of its lysine residues,[9,26−32] that trypsin would be unable to hydrolyze the peptide bond C-terminal to a lysine residue adducted by **4**. The simplest and commonest consequence of a missed cleavage at a trypsin-resistant site is, in effect, to merge two consecutive peptides by shifting the C-terminal cleavage to the next lysine or arginine. Thereby the tryptic peptides obtained from a modified protein either differ substantially from those obtained from the native protein or have no counterparts.[32] By this method, eight and seven adducted peptides, all products of single missed cleavages at lysine residues, were found using nanoLC-MS/MS in digests of the modified HSA and RSA, respectively (Tables 3 and 4).

For example, modification of lysine-199 in HSA merged the theoretical dipeptide LK (residues 198−199), which is not detected in digests of unmodified HSA,[32] with CASLQK to give peptide MT3(H). This particular effect on tryptic hydrolysis has been obtained previously by acylation of lysine-199 with acyl glucuronides,[9,27] *O*-acyl ureas,[28] a coenzyme A thioester,[30] a carboxylic anhydride,[29] and benzylpenicillin.[26] An analogous modified neopeptide, MT2(R), was obtained from RSA. Confirmation of the modification sites by collision-induced (MS/MS) sequencing was not possible because every acylated peptide gave only [MH$^+$] for the unmodified peptide as a product ion and/or the corresponding *b*- and *y*-series sequence fragments. Evidently the *N*ϵ-lysylamide linkages were invariably weaker than the peptide bonds. However, except for three peptides [(MT1(H), MT5(H), and MT5(R)] that yielded only one of the nonsequence fragments, additional evidence for the proposed acylations was provided by two nonsequence product ions

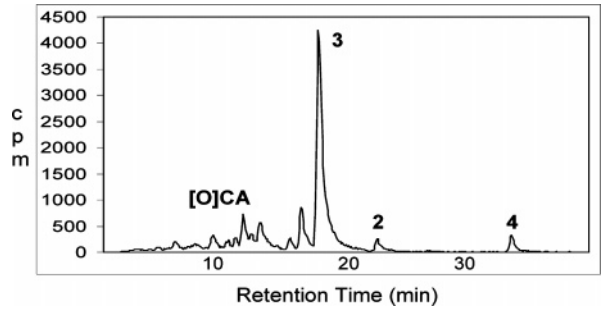


Figure 5. Radiochromatogram (reversed-phase HPLC) of the biliary metabolites (0−1 h fraction) of [^{14}C]**2** (15 μmol/kg; labeled at C-2 of the thiadiazole ring) in an anesthetized and cannulated adult male rat. Bile was collected over glacial acetic acid to stabilize acyl glucuronides. [O]CA = hydroxylated carboxylic acids (t_R 10−17 min). Acyl glucuronide **3**, carboxylic acid **2**, and imide **4** were identified by comparison (LC-MS) with authentic standards.

attributable to a glutaramide moiety: *m*/*z* 322 ([MH$^+$] for **4**) and *m*/*z* 130 (thiadiazole ring).

Metabolism of [^{14}C]2 and Cyclic Imide 4 in Rats. Anaesthetized and cannulated adult male rats administered [^{14}C]**2** (15 μmol/kg; iv) labeled at C-2 of the thiadiazole ring eliminated 28.7 ± 5.3% and 75.1 ± 8.4% (mean ± SD, n = 4) of the radiolabel in bile over 1 and 5 h, respectively. Urine removed from their bladders after 5 h contained 4.9 ± 3.1% of the dose. Plasma samples obtained at the same time contained only 0.3−0.4% of the dose per mL. When bile was collected hourly without the acidification that stabilizes acyl glucuronide **3**, the radiolabeled material was found to be comprised principally of carboxylic acids **2** and **5** (50% and 15% of chromatographed radioactivity, respectively, in 0−1 h bile fraction), identified by LC-MS, and a mixture of six or seven hydroxycarboxylic acids (27% collectively, ca. 1−15% individually; t_R 10−17 min; *m*/*z* 356 for [MH$^+$]). Except for the least polar isomer, which yielded *m*/*z* 146 (130 + [O]) for the functionalized heterocycle, all of the hydroxycarboxylic acids yielded *m*/*z* 130 and thereby were modified outside the thiadiazole ring (Table 2). Imide **4** (6%) and traces of dihydroxycarboxylic acids (t_R 5.5−8.5 min; *m*/*z* 372) were also found in bile but no trace of acyl glucuronide **3**. The hydroxylated and dihydroxylated metabolites were those found previously in the plasma of rats administered **2**.[2] Bile fully acidified by collection onto glacial acetic acid (final acid concentration in the 1-h fraction, ca. 300 mM) contained substantial amounts of **3** (65% chromatographed radioactivity; 0−1 h collection) and only small amounts of **4** (7%) and **2** (4%) (Figure 5). Acid **5** was not seen in the first two hourly bile collections and never accounted for more than approximately 1% of the radioactivity in later collections, suggesting any **4** produced within hepatocytes was not hydrolyzed extensively, or if it was hydrolyzed, that **5** was transported selectively into blood. As the hydroxylated and dihydroxylated carboxylic acids were found in acidified bile from the first hour, it is likely they were metabolites of **2** rather than both **2** and **5**. In later bile fractions, a fall in the proportions of **3** was counterbalanced principally by a rise in the hydroxycarboxylic acids rather than in **4**, **2**, or **5**. Bile partially acidified by collection onto 10% (v/v) acetic acid contained substantially less **3** (27−45%; 0−1 h collection) and correspondingly larger amounts of **4** (21−35%) but still contained only small proportions of acids **2** (6−9%) and **5** (1−2%); emphasizing the slowness of imide hydrolysis relative to glucuronide cyclization. Acyl glucuronide **3** was hydrolyzed completely to **2,** without formation of **4** or **5**, when fully acidified bile was incubated with *H. pomatia* β-glucuronidase (Sigma) in 0.1 M sodium acetate, pH 5.0, at 37 °C for 3 h. This in itself indicates **3** had not undergone acyl migration because C-2, -3, and -4 acyl glucuronides are typically resistant to β-glucuronidase.[33] The rapid degradation of **3** in rat bile, which has a pH of approximately 7.5,[34] was confirmed by incubating synthetic glucuronide (2 mM) in pre-dose bile at 37 °C: after 1 h, it had degraded completely to **4**, **2**, and **5** (ratio **2**:**5**, 0.9). Even in fully acidified bile at 5 °C, **3** underwent extensive degradation to **4**, **2**, and **5**, which was complete by 18 h. The only identified radiolabeled compounds in (unacidified) urine were the hydroxylated carboxylic acids, which even in fully acidified bile were never found as glucuronides.

Imide **4** (50 μmol/kg) given iv to cannulated rats persisted to the extent that 4.5 ± 1.9% and 11.0 ± 4.3% (mean ± SD, n = 3) of the dose was recovered unchanged in bile over 1 and 5 h, respectively. Acids **2** and **5** and several of the hydroxylated carboxylic acids that were metabolites of [^{14}C]**2** were also found in bile. Only trace amounts of **4** were detected in bladder urine by LC-MS at 5 h.

Discussion

The unusual instability of acyl glucuronide **3** is emphasized by comparing the conjugate's first-order half-life in phosphate buffer, pH 7.4, at 37 °C (10.2 min) with the half-lives under similar conditions of 31 structurally diverse acyl glucuronides (0.26−79 h), 19 of which have half-lives ≥100 min and only six-half-lives ≤30 min.[4] None of these glucuronides is known to degrade in aqueous solution except by acyl migration and hydrolysis. The most reactive of them, tolmetin glucuronide ($t_{1/2}$ 16 min), irreversibly modifies human plasma protein in vivo[35] and in vitro,[27,36] but the mechanism of degradation of **3** is so fundamentally different it is not possible to predict an extent of protein modification from degradation rates, as it is when comparing conventionally unstable acyl glucuronides.[36] While it may be presumed that the rapidity of intramolecular nucleophilic amidolysis simply precludes a slower intramolecular migration, the complete stability of *N*-propyl **3** (acyl glucuronide **11**) in phosphate buffer, pH 7.4, at 37 °C over 24 h implies **3** is intrinsically resistant to acyl migration. One potential explanation is the bulky substitution pattern proximate to the aglycone's acid moiety: steric hindrance by alkyl substituents at the α- and β-carbons has been invoked to rationalize the slower rearrangement of certain acyl glucuronides.[4]

A recent ^{1}H NMR study of the spontaneous degradation of several acyl glucuronides at pH 7.4 and 37 °C reported only very rapid hydrolysis in the case of **3**.[37] Consequently, it was concluded that glucuronide metabolites such as **3** are likely to be relatively unreactive in biological systems and thereby not significant liabilities in drug development, because a dominant hydrolysis process will minimize the (acyl migration) pathway known to produce intermediates that retain the ability to react covalently with proteins.[4,6,7] The present finding that **3** cyclizes to an electrophilic imide under these conditions emphasizes that only a detailed deconstruction of an exceptional reactivity will reveal the full potential for biochemical interactions.

The great rapidity of the cyclization of **3** except under unphysiologically acidic conditions, which were essential for definitive identification of the acyl glucuronide in bile, implies that the conjugate may degrade partially within the liver and is certain to degrade quantitatively in the alkaline conditions of the small intestine. Therefore the mixture of carboxylic acids and imide **4** found in unacidified bile may resemble the metabolites to which the intestine is exposed in animals administered carboxylic acid **2**.

The only documented rearrangement of acyl glucuronides bearing any resemblance to cyclization of **3** is a rapid cyclization

at pH values >7 that forms δ-lactones from biosynthetic glucuronides of statin derivatives with 2,4-dihydroxy heptanoic acid side chains.[38] As was the case with **3**, the glucuronide of hydroxy simvastatin does not appear to form acyl migration products and degrades rapidly to the δ-lactone following its elimination in rat bile. The biosynthetic acyl-CoA thioester of hydroxy simvastatin undergoes an analogous lactonization.[39]

An alternative mechanism of cyclization of **3** involving nucleophilic attack by the amide carbonyl rather than the nitrogen, with consequential loss of the heterocycle, would generate initially an imino-ether and then a substituted glutaric anhydride, and finally, upon hydrolysis, 2-cyclopentyl-4-propyl-glutaric acid. This mechanism is analogous to the intramolecular reaction between the carboxamide and epoxide functions of allylisopropylacetamide epoxide, which produces a stable γ-butyrolactone, hypothetically via an electrophilic iminolactone.[40] Nevertheless, the extensive degradation of **3** in aqueous solution at pH 7.4 did not yield any 2-cyclopentyl-4-propyl-glutaric acid detectable by LC-MS.

Notwithstanding the rapid cyclization of **3** under physiological conditions and the reactivity of imide **4**, contributory conjugate formation by direct reactions between **3** and amine or thiol nucleophiles cannot be excluded. However, this is unlikely to have a material effect because the amide and thioester conjugates obtained from **3** were identical to those of **4.** Simple carboxylic acids can be eliminated by rats as thioester conjugates,[41,42] but the instability of thioesters **18a**/**18b** and their glutathione analogues at pH 7.4 implied that no thioester conjugates of acids **2** or **5** produced in vivo, by either direct reaction or transacylation of the abundant metabolite **3**,[41] would be sufficiently persistent to allow detection in biological media ex vivo. In fact, using the synthetic conjugates of cysteine, lysine, Nα-acetylated cysteine and lysine, and glutathione as standards, no thioester or amide metabolites of either [^{14}C]**2** or **4** were found in rat bile or urine.

Serum albumins, and particularly HSA, which has 125 nucleophilic side chains, are well-documented targets for low molecular weight electrophiles. HSA and RSA, when incubated with the highest concentration of **4** (500 μM), underwent Nε-acylations of lysine residues that were abundant and stable enough to be characterized in tryptic peptides of the denatured and reduced proteins. From the reactions of **4** with *N*α-acetyl L-lysine, it is likely the adducts were *N*ε-lysylamides of both **5** and **2**. The absence of an acylated cysteine residue was predictable from the instability of *N*α-acetyl L-cysteine conjugates (**18a** and **18b**) and glutathione conjugates of **2** and **5** and conforms with the behavior of hexahydrophthalic anhydride, which produces stable derivatives of HSA lysines but not cysteine-34.[24,29] All eight of the modified HSA lysines are known, variously, as sites of acylation by acyl glucuronides,[9,27] a coenzyme A thioester,[30] a carboxylic anhydride,[29] and benzylpenicillin[26] in vitro. However, only lysines-195, -199, and -541 are commonly encountered as targets of acylation, and perhaps only lysine-199, which has a pK_a estimated to be 7.47,[43] is almost invariably modified. It is also the site of acetylation by aspirin[44] and a lesser site of glycation by glucose.[45] Albeit from a very limited database,[9,26−32] the set of eight HSA lysines modified by **4** most closely resembles the six adducted by tolmetin acyl glucuronide (five residues are modified by both compounds).[27] Selective derivatization of RSA lysines has not been characterized in comparable detail. The fraction of lysines modified by **4** is similar: seven (RSA) and eight (HSA) from 53 and 59 residues, respectively. Of the 40 homologous lysines (Swiss-Prot database) six and five in RSA and HSA, respectively, are acylated; four are common to both proteins. Predicting in vivo protein modifications from in vitro data is currently highly problematical. Limited studies on benzylpenicillin indicate the same set of HSA lysines is modified in vitro and in vivo,[26] and there is at least a partial overlap in the case of glucose.[45] Protein modification by **4** in vivo remains a possibility: the occurrence of **5** as the major plasma metabolite of **2**[20,46] in itself implies formation of **4** in vivo; **4** has been found as a urinary metabolite and minor plasma metabolite of **2.**[46] The present studies have shown that the imide can pass from blood into and through the liver in rats.

Conclusion

Although the generic instability of acyl glucuronides has been analyzed in great detail,[4] glucuronide **3** of glutaramide **2** has now been shown to undergo an unprecedented and rapid eliminative cyclization (decomposition half-life, 10.2 min) to the apparent exclusion of the familiar intramolecular acyl migration. Reactivity of **3** was characterized using glucuronide prepared efficiently by selective acylation of benzylglucuronate with *N*-Bom-protected **2** obtained by a three-step, one-pot synthesis. The association between exceptional acyl glucuronide instability and a combination of low amide pK_a and formation of a stable six-membered ring immediately suggested structural modifications for stabilizing glucuronides of series **1** glutaramides by retarding the intramolecular nucleophilic amidolysis. Pryde et al. [1b,5] have shown that monocarboxylic glutaramides possessing nonaromatic amide substituents have higher NH pK_a and yield acyl glucuronides with greatly enhanced stability. A potent NEP inhibitor from this second group, the 4-chlorophenpropylamide analogue of **2** (*S*-**30**), having an estimated pK_a of 16,[5b] was selected as a clinical candidate[1b]. The decomposition half-life of *S*-**30** in phosphate buffer, pH 7.4, at 37 °C is 51 h;[46] the longest documented half-life of an acyl glucuronide under comparable conditions is the exceptional 79 h of the valproic acid conjugate.[4] The decomposition half-life of **3** in rat, dog, and human blood ex vivo at 37 °C is approximately 5 min[1b], whereas the acyl glucuronide of *S*-**30**, under these conditions, does not degrade for at least 1 h[1b]. Additionally, the cyclic imide of *S*-**30**, unlike **4**, is resistant to hydrolysis at pH 7.4 and does not react spontaneously with glutathione in aqueous solution to form a thioester.[46] While it is altogether premature to draw any conclusions with respect to a possible causal linkage between the stability/reactivity of these glutaramide acyl glucuronides and the parent drugs' toxicity in vivo, it is notable that *S*-**30** is free of the acute intestinogastric toxicity, including especially hemorrhagic necrosis of the mucosal epithelium, that was associated with **2** in dogs.[5b,46]

Experimental Section

Chemical Procedures. Organic extracts were washed finally with satd aq NaCl and dried over anhydrous Na_2SO_4 prior to rotary evaporation at <30 °C. Analytical thin-layer chromatography was performed using Merck Kieselgel 60 F 254 silica plates. Preparative column chromatography was performed on Merck 938S silica gel. Rotations were measured on an Optical Activity polarimeter operating at the wavelength of the sodium D-line. Infrared spectra were recorded using a Perkin-Elmer RX1 FTIR instrument, for the physical forms noted. Unless otherwise stated, ^{1}H and ^{13}C NMR spectra were recorded on $CDCl_3$ solutions using either Bruker 250 or 400 MHz (100 MHz for ^{13}C) instruments with tetramethylsilane as internal standard. Both low- and high-resolution mass spectra were obtained by direct injection of sample solutions into a Micromass LCT mass spectrometer operated in the electrospray mode, +ve or −ve ion as indicated. CI mass spectra (NH_3) were obtained on a Fisons Instruments Trio 1000. The pK_a of acid **2** were

determined at 20 °C by pH-metric titration using a GL pK_a titrator (Sirius Analytical Instruments, Forest Row, East Sussex, UK). Duplicate measurements were performed with **2** (0.5 mM) in H_2O/MeOH/DMSO (60:30:10, v/v).

(3*S*,4*S*,5*S*,6*S*)-2-Propenyl 6-[(*R*)-2-[(1-(5-ethyl-1,3,4-thiadiazol-2-yl)carbamoyl]cyclopentyl]methyl]pentanoyloxy]-3, 4, 5-trihydroxytetrahydro-2*H*-pyran-2-carboxylate (7). Via the Mitsunobu Reaction. DIAD (0.10 mL, 0.5 mmol) was added over 10 min to a solution of (2R)-2-[(1-{[(5-ethyl-1,3,4-thiadiazol-2-yl)amino]-carbonyl}cyclopentyl)methyl]pentanoic acid **2** (0.170 g, 0.5 mmol), triphenylphosphine (0.135 g, 0.5 mmol), and allyl-α,*β*-D-glucuronate **6** (0.12 g, 0.5 mmol) in anhydrous THF (3 mL) which was stirred under N_2 at 0 °C. After 1 h the solution was evaporated to dryness and then purified first by silica chromatography, eluting with 5% MeOH in CH_2Cl_2. Appropriate fractions were pooled and evaporated to give the product as an α/*β* mixture. Further purification by preparative HPLC (Luna 10 *μ*m C-18 column, 250 × 21.2 mm, eluting with 30−50% MeCN in H_2O over 30 min) afforded on evaporation of appropriate fractions (t_R = 29.3 min) the desired **7** as a single *β*-anomer (foam, 0.081 g, 30%); ^{1}H NMR (400 MHz, CD_3OD): 0.84 (3 H, t, *J* = 7.3 Hz), 1.4−1.7 (13 H, m), 2.00 (1 H, dd, *J* = 13.8 and 3.2 Hz, 1′-H), 2.20−2.40 (3 H, m), 3.09 (2 H, q, *J* = 7.3 Hz), 3.40−3.60 (3 H, m, 3-H, 4-H and 5-H), 4.00 (1 H, d, *J* = 9.7 Hz, 2-H), 4.68 (2 H, d, *J* = 5.4 Hz), 5.30 (2 H, m, CH_2═CHCH$_2$), 5.45 (1 H, d, *J* = 7.8 Hz, 6-H) and 5.75−6.09 (1 H, m, CH$_2$═*CH*CH$_2$); ^{13}C NMR (100 MHz, CD_3OD) 13.5, 19.8, 22.8, 23.4, 23.8, 33.6, 34.2, 35.6, 36.9, 39.9, 42.5, 55.1, 65.4, 71.4, 72.1, 75.7, 76.1, 94.3, 117.4, 132.0, 159.4, 167.0, 168.1, 174.8 and 175.7. LC-MS (30−50% MeCN in 5 mM NH_4OAc, 20 min; t_R = 15.5 min); *m*/*z* (ES +ve mode) 556 ([MH$^+$], 100%).

General Procedure for Acyl Glucuronide Formation by Selective Acylation. A mixture of glucuronate monoester **6** or **13** (0.5 mmol), carboxylic acid (0.5 mmol), HATU (0.19 g, 0.5 mmol), and 1,4-diazabicyclo[2.2.2]octane (0.112 g, 1 mmol) or NMM (0.11 mL, 1 mmol) in anhydrous MeCN (5 mL) was stirred under nitrogen at 20 °C. The reaction was monitored by TLC (10% EtOH−CH_2Cl_2), and after 3 h it was quenched by adding Amberlyst A-15 (H$^+$, 1 mmol) and stirring for 30 min. The beads were filtered off, and the residue plus an MeCN washing was evaporated to dryness followed by chromatography, eluting with 7% EtOH−CH_2Cl_2. Appropriate fractions were concentrated to give the product **10** or **14**.

(3*S*,4*S*,5*S*,6*S*)-2-Propenyl 6-[(*R*)-2-[[1-(2-Propenyl)(5-ethyl-1,3,4-thiadiazol-2-yl)carbamoyl]cyclopentyl]methyl]pentanoyloxy]-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-carboxylate (10). This was prepared by the general method and obtained as a foam in 65% yield; ^{1}H NMR (400 MHz, CD_3OD) 0.79 (3H, t, *J* = 7.3 Hz), 1.20−1.60 (13 H, m), 1.96 (1 H, dd, *J* = 13.8 and 3.2 Hz), 2.10 (1 H, dd, *J* = 13.8 and 9.0 Hz), 2.26 (1 H, m), 2.32 (1 H, m), 2.92 (2 H, m), 3.30−3.70 (3 H, 3 m, 3-H, 4-H and 5-H), 4.02 (1 H, d, *J* = 9.5 Hz, 2-H), 4.65 (2 H, m), 4.94 (2 H, m), 5.17−5.37 (4 H, m, CH_2═CHCH$_2$), 5.50 (1 H, d, *J* = 8.1 Hz, 6-H) and 5.75−6.09 (2 H, m, CH$_2$═*CH*CH$_2$); ^{13}C NMR (100 MHz, CD_3OD) 12.2, 13.4, 20.0, 23.7, 23.8, 24.7, 33.7, 36.1, 39.0, 41.6, 42.9, 52.4, 56.4, 65.1, 71.7, 72.7, 76.0, 76.2, 94.4, 117.1, 117.9, 131.6, 132.2, 160.3, 165.1, 167.9, 174.8 and 186.4. *m*/*z* (ES +ve mode) 618 ([MNa$^+$], 100%). High-resolution mass (ES +ve mode) calculated for $C_{28}H_{41}N_3O_9$SNa ([MNa$^+$]): 618.2461. Found: 618.2431.

(*R*)-2-[[1-[Benzyloxymethyl](5-ethyl-1,3,4-thiadiazol-2-yl)carbamoyl)]cyclopentyl]methyl]pentanoic Acid (12). A solution of TBSCl (0.393 g, 2.5 mmol) in CH_2Cl_2 (2 mL) was added to a solution of compound **2** (0.339 g, 1 mmol) in CH_2Cl_2 (5 mL) and triethylamine (0.35 mL. 2.5 mmol) which had been stirred for 0.5 h at 25 °C. After 20 h, Bom-Cl (0.232 mL, 1 mmol) was added and stirring was continued for another 6 h. The solvents were evaporated, and the residue was redissolved in THF (10 mL) followed by addition of 1 M TBAF in THF (2.5 mL) at 0 °C. After 1 h water (20 mL) was added followed by extraction with EtOAc (3 × 15 mL). The combined organic extracts were washed with satd aq $NaHCO_3$, and then standard workup followed by evaporation afforded the product **12** as an oil (0.367 g, 80%); Anal. ($C_{24}H_{33}N_3O_4S$) C, H, N. ^{1}H NMR (400 MHz, $CDCl_3$): 0.83 (3 H, t, *J* = 7.2 Hz), 1.21−1.65 (13 H, m), 1.95 (1 H, dd, *J* = 13.8 and 3.2 Hz), 2.17 (1 H, dd, *J* = 13.9 and 9.0 Hz), 2.26 (2 H, m), 2.88 (2 H, m), 4.73 (2 H, m, Ph*CH*$_2$O), 5.75 (2 H, s, NCH$_2$O) and 7.35 (5 H, m, ArH); *m*/*z* (CI, NH_3) 460 ([MH$^+$], 100%).

(3*S*,4*S*,5*S*,6*S*)-6-[(*R*)-2-[[1-(5-Ethyl-1,3,4-thiadiazol-2-yl)carbamoyl]cyclopentyl]methyl]pentanoyloxy]-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-carboxylic Acid (3). A mixture of compound **14** (0.142 g, 0.2 mmol; see Supporting Information) and 10% Pd−C (0.106 g) in glacial AcOH (4 mL) was stirred under an H_2 balloon at 20 °C for 18 h. The mixture was filtered through Celite, the solids were washed with a small quantity of AcOH, then the combined filtrate and washings were evaporated to give a colorless oil which was triturated with acetone to afford the product **3** as a white solid (0.070 g, 70%); ^{1}H NMR (400 MHz, CD_3OD): 0.83 (3 H, t, *J* = 7.1 Hz), 1.20−1.61 (13 H, m), 2.00 (1 H, dd, *J* = 13.8 and 3.2 Hz), 2.17 (1 H, dd, *J* = 13.9 and 9.0 Hz), 2.26 (2 H, m), 2.88 (2 H, m), 3.30−3.50 (3 H, 3 m, 3-H, 4-H and 5-H), 3.65 (1 H, brs, 2-H) and 5.43 (1 H, d, *J* = 7.5 Hz, 6-H); ^{13}C NMR (100 MHz, CD_3OD) 14.4, 21.3, 23.2, 24.9, 25.2, 25.4, 26.7, 35.9, 36.8, 37.7, 38.7, 41.2, 44.1, 56.4, 73.4, 73.9, 77.9, 95.8, 169.1, 176.4, 177.7, 179.8 and 179.8; LC-MS, single peak (20 to 50 to 70% MeCN in 5 mM NH_4OAc, 30 and 5 min, respectively; t_R = 18.5 min); *m*/*z* (ES +ve mode) 516 ([MH$^+$], 100%), (ES-ve mode) 514 [M − H]$^-$. High-resolution mass (ES-ve mode) calculated for $C_{22}H_{32}N_3O_9S$ [M − H]$^-$ 514.1859. Found: 514.1843.

(*R*)-7-(5-Ethyl-1,3,4-thiadiazol-2-yl)-9-propyl-7-azaspiro[4.5]-decane-6,8-dione (4). *N*-Methylmorpholine (0.066 mL, 0.6 mmol) and DIC (0.1 mL, 0.66 mmol) were added successively to a mixture of compound **2** (0.203 g, 0.6 mmol) and 1-hydroxybenzotriazole (0.027 g, 0.2 mmol) in CH_2Cl_2 (3 mL) which was stirred at 0 °C. The resulting solution was allowed to attain 20 °C, and then after 1 h it was concentrated and directly purified by chromatography, eluting with 20% EtOAc−hexane. Evaporation of appropriate fractions afforded the product **4** as a white solid (0.160 g, 83%); Anal. ($C_{16}H_{23}N_3O_2S$) C, H, N. ^{1}H NMR (400 MHz, $CDCl_3$) 0.96 (3H, t, *J* = 7.2 Hz), 1.45−1.60 (7 H, m), 1.65−2.10 (9H, m), 2.47 (1H, m), 2.77 (1H, m) and 3.16 (2H, q, *J* = 7.0 Hz); *m*/*z* (CI, NH_3) 322 ([MH$^+$]).

Isomeric Acid 5. *R*-1-(2-*tert*-Butoxycarbonylpentyl)cyclopentanecarboxylic acid[1] (513 mg, 1.80 mmol) was dissolved in methanol (5 mL), and cesium carbonate (294 mg, 0.9 mmol) added in one portion with stirring. After 5 min, the mixture was evaporated to low volume and azeotroped with toluene (2 mL). The residue was redissolved in DMF (5 mL) under nitrogen, benzyl bromide (225 *μ*L, 1.83 mmol) added in one portion, and the whole stirred at room temperature for 3 h. The mixture was poured into EtOAc (40 mL) and washed with water (40 mL), 1 M HCl (20 mL) and water again (20 mL). The resulting solution was dried over $MgSO_4$ and evaporated to a thick oil, which was purified by column chromatography using pentane−EtOAc (2:1, v/v) as eluent to provide 430 mg of a clear oil. This was dissolved in TFA (2 mL), left at room temperature for 16 h, and then evaporated to dryness in vacuo to provide 353 mg of a thick oil. This oil was dissolved in acetonitrile (5 mL), and 2-amino-5-ethyl-1,3,4-thiadiazole (150 mg, 1.15 mmol), ethyl dimethylaminopropylcarbodiimide HCl (255 mg, 1.23 mmol), 1-hydroxybenzotriazole (173 mg, 1.23 mmol), and *N*-methylmorpholine (243 *μ*L) were added in sequence. The whole was stirred at room temperature for 16 h and at 80 °C for 3 h. It was allowed to cool to room temperature, evaporated to dryness, redissolved in EtOAc (10 mL), and washed with saturated $NaHCO_3$ solution. The organics were dried over $MgSO_4$, filtered, and evaporated to a thick oil, which was purified by column chromatography using pentane−EtOAc (2:1, v/v) to provide 430 mg of a clear oil. This oil was dissolved in EtOH−MeOH (5:1, v/v), palladized charcoal (45 mg) added and the whole stirred under hydrogen (30 psi) for 2 h. The catalyst was removed with a short plug of celite, and the solution was evaporated to a clear oil. Column chromatography using a gradient of DCM in MeOH (19:1 to 9:1, v/v) provided the title compound (120 mg, 20%) as a clear oil. Anal. ($C_{16}H_{25}N_3O_3S$) C, H, N. ^{1}H NMR (400 MHz, $CDCl_3$): 0.89 (3H, t, *J* = 6.4 Hz), 1.16−1.86 (13H, m, including 1.38, 3H, t, *J*

= 7.5 Hz), 1.91−2.03 (1H, m), 2.22−2.40 (1H, m), 2.42−2.73 (2H, m), 2.95 (2H, dq, J = 7.5, 2.4 Hz); m/z (ES+ve mode) 340 ([MH^+]).

***N*α-Acetyl L-Lysine Adducts (17a, 17b).** A mixture of imide **4** (0.128 g, 0.4 mmol) and *N*α-acetyl L-lysine **15** (0.150 g, 0.8 mmol) was stirred in EtOH (2 mL) and sodium tetraborate buffer, pH 9.22 ± 0.02, (BDH buffer tablet, 25 mM $Na_2B_4O_7$ with 1.7 mM NaCl; 2 mL) at 37 °C for 24 h. HCl (1 M) was added to give a pH of 3, and then the product was extracted with EtOAc (3 × 15 mL) and worked up in the usual manner. The crude product was separated by preparative HPLC (Luna 10 μm C-18 column, 250 × 21.2 mm, eluting with 30 to 50 to 70% MeCN in 0.1% aq formic acid over 20 and 10 min, respectively) to afford **17a** and **17b** (combined yield 60%) having t_R 29.9 and 31.0 min, respectively. For **17a** (major isomer; amide of **2**): Anal. ($C_{24}H_{39}N_5O_5S\cdot 2H_2O$) C, H, N. ^{1}H NMR (400 MHz, CD_3OD) 0.74 (3H, t, J = 7.1 Hz), 1.05−1.42 (11H, m), 1.45−1.62 (7H, m), 1.72 (2H, m), 1.89 (3H, s, CH_3CO), 2.02−2.22 (4H, m), 2.81−2.98 (4H, m) and 4.24 (1H, m, NH*CH*CO_2H); LC-MS (20 to 50 to 70% MeCN in 0.1% aq formic acid, 30 and 5 min, respectively) t_R 16.6 min; m/z (ES+ve mode) 510 ([MH^+], 100%). For **17b** (minor isomer; amide of **5**): ^{1}H NMR (400 MHz, CD_3OD) 0.80 (3H, t, J = 7.3 Hz), 1.12−1.60 (18H, m), 1.70 (2H, m), 1.87 (4H, m), 1.99 (1H, m), 2.10 (1H, m), 2.45 (1H, m) 2.90−3.15 (4H, m) and 4.24 (1H, m, NH*CH*CO_2H); LC-MS (20 to 50 to 70% MeCN in 0.1% aq formic acid, 30 and 5 min, respectively) t_R 19 min; m/z (ES+ve mode) 510 ([MH^+], 100%). Ratio **17a** to **17b** (254 nm): 3.0.

***N*α-Acetyl L-Cysteine Adducts (18a, 18b).** Triethylamine (0.17 mL, 1.2 mmol) was added to a mixture of imide **4** (0.096 g, 0.3 mmol), *N*-acetyl L-cysteine **16** (0.098 g, 0.6 mmol) and a trace of 4-dimethylaminopyridine in anhydrous MeCN (3 mL) with stirring at 20 °C. After 4 h, when reaction appeared complete by TLC (60% EtOAc−hexane), the mixture was evaporated to dryness and purified by preparative HPLC (Luna 10 μm C-18 column, 250 × 21.2 mm, eluting with 50−70% MeCN in 0.1% aq formic acid over 20 min) to afford **18a** and **18b** (combined yield 42%, ratio 3:1, w/w) having t_R 13.5 and 10.3 min, respectively. For **18a** (major isomer; thioester of **2**): ^{1}H NMR [400 MHz, $(CD_3)_2CO$] 0.82 (3H, t, J = 7.4 Hz), 1.26−1.72 (13H, m), 1.96 (3H, s, CH_3CO), 2.03 (1H, s), 2.32−2.43 (3H, m), 2.60 (1H, m), 3.01−3.18 (3H, m), 3.43 (1H, m) and 4.65 (1H, m, NH*CH*CO_2H); LC-MS (20 to 50 to 70% MeCN in 0.1% aq formic acid, 30 and 5 min, respectively), t_R 27.5 min; m/z (ES+ve mode) 485 ([MH^+], 100%). High-resolution mass (ES-ve mode) calculated for $C_{21}H_{31}N_4O_5S_2$ [$M - H]^-$: 483.1736. Found: 483.1749. For **18b** (minor isomer; thioester of **5**): ^{1}H NMR [400 MHz, $(CD_3)_2CO$] 0.90 (3H, t, J = 7.2 Hz), 1.22−1.68 (13H, m), 1.98 (2H, m), 2.10−2.20 (2H, m), 2.30 (3H, s, CH_3CO), 2.70 (1H, m), 3.04 (2H, q, J = 7.0 Hz), 3.19 (1H, dd, J = 13.9 and 8.0 Hz), 3.46 (1H, dd, J = 13.9 and 4.6 Hz) and 4.82 (1H, m, NH*CH*CO_2H); LC-MS (20 to 50 to 70% MeCN in 0.1% aq formic acid, 30 and 5 min, respectively), t_R 23.4 min; m/z (ES+ve mode) 485 ([MH^+], 100%). Ratio **18a** to **18b** (254 nm): 2.9.

Stability of Acyl Glucuronide 3 and Cyclic Imide 4 in Aqueous Solution. Degradation half-lives of **3** (39 μM) and **4** (62 μM) in 100 mM sodium dihydrogen phosphate buffer, pH 7.4, were determined at 37 °C. Aliquots (100 μL) were removed at 10-min intervals for 60 min and thereafter at 90 min, 3, 5, 7, and 27 h, and mixed immediately with 1% trifluoroacetic acid (20 μL). Acidified solution (80 μL) was eluted from a Hypersil HS C-18 column (50 mm × 4.4 mm; Thermo Fisher Scientific, Waltham, MA) with a gradient of acetonitrile (25% for 3 min, 25% to 90% over 6 min, 90% for 5 min) in 4 mM ammonium formate, pH 3.5, delivered by a Shimadzu 10A HPLC system (Shimadzu UK, Milton Keynes, Buckinghamshire, UK). **3** (3.8 min), **2** (8.8 min), **5** (9.4 min), and **4** (11.5 min) were detected at 254 nm and identified by cochromatography with authentic compounds and matching of electrospray product-ion mass spectra. Peak areas of **3** and **4** (λ = 254 nm) in sequential aliquots were integrated and subjected to linear regression analysis (r^2 = 0.99) to obtain degradation half-lives ($t_{1/2}$ = ln2/slope). The absence of acyl migration during degradation of **3** (39 μM) in deuterated phosphate buffer (100 mM), pD ca. 7.2, at 20 °C was confirmed by NMR spectroscopy using ^{1}H and 1D and 2D TOCSY methods. Data were acquired on a Varian Unity Inova 500 MHz spectrometer equipped with a Narolac 3-mm inverse detection probe.

Reactions of Acyl Glucuronide 3 and Cyclic Imide 4 with Amino Acids and Glutathione. Acyl glucuronide **3** (2 mM) and either *N*α-acetyl L-lysine **15**, *N*-acetyl L-cysteine **16**, or glutathione (80 mM) in 0.5 mL of 100 mM sodium phosphate, pH 7.4, were incubated at 37 °C for 1−20 h in 1.5 mL polypropylene microtubes. Imide **4** (3 mM; 1.0 mL) and either glutathione, glutathione disulfide, or an amino acid (120 mM; Table 1) were incubated under the same conditions. Aliquots (50−100 μL) were removed at intervals and analyzed by LC-MS.

Covalent Modification of Serum Albumin by Cyclic Imide 4. Imide **4** (final concentration, 5, 50, or 500 μM) dissolved freshly in either DMSO or acetonitrile (5 μL) was added to a solution (1 mg/mL; 15 μM; 0.5 mL) of either HSA or RSA (approx 99% pure, essentially globin free and fatty acid free; Sigma, UK) in 100 mM sodium phosphate, pH 7.4. The mixture was incubated in polypropylene microtubes at 37 °C for 16 h. An aliquot (50 μL) was taken for LC-MS analysis. Disulfide bridges in a second aliquot of protein were reduced with dithiothreitol (final concentration, 1 mM; room temperature, 15 min) and the free thiols carboxyamidomethylated with iodoacetamide (final concentration, 55 mM; room temperature, 15 min). The solution of protein thus modified (1 μL) was diluted with 50 mM ammonium hydrogen carbonate, pH 7.6, (1:10, v/v) and digested with 50 ng of sequencing grade modified trypsin (Promega UK, Southampton, Hampshire, UK) at 37 °C for 16 h. The tryptic digest (3 μL injection volume; equivalent to 0.3 μg of protein) was chromatographed on a PepMap 3 μm C-18 Nano Series column (15 cm × 75 μm; LC Packings, Amsterdam, Netherlands) preceded by a 5 mm C18 μ-precolumn cartridge. A gradient from 5% acetonitrile/0.05% TFA (v/v) to 48% acetonitrile/0.05% TFA (v/v) in 60 min was applied at a demanded flow rate of 200 nL/min using an UltiMate capillary LC system (Dionex Corp, Sunnyvale, CA). Eluted peptides were delivered to a QSTAR Pulsar i mass spectrometer (Applied Biosystems, Foster City, CA) via a 10 μm i.d. PicoTip nanoelectrospray emitter (New Objective, Woburn, MS), and spectra were acquired in positive-ion mode through information-dependent acquisitions controlled by Analyst software as described previously.[47]

Metabolism of [^{14}C]2 and Cyclic Imide 4 in Rats. Adult male Wistar rats were anesthetized with urethane (1.4 mg/mL in isotonic saline; 1 mL/kg, ip). Cannulae were inserted into the trachea, jugular vein, and common bile duct, and the penis was ligated. Either [^{14}C]-**2** (15 μmol/kg, 10 μCi; in 200 μL of DMSO) radiolabeled at C-2 of the thiadiazole ring (18 mCi/mmol; radiochemical purity, 98% as determined by HPLC) or **4** (50 μmol/kg; in 300 μL of DMSO), both dissolved in DMSO immediately before administration, was injected over 20 min via the jugular vein. Bile was collected into polypropylene microtubes hourly for 5 h. When rats were given [^{14}C]**2**, the bile fractions were in some cases acidified as they collected in order to stabilize acyl glucuronide metabolites for characterization. For acidification, the tubes contained either 15 μL of glacial acetic acid or 10 μL of water−acetic acid (9:1, v/v). Urine was removed from the bladder at the end of each 5-h experiment without acidification, and a blood sample was taken at the same time. Bile (20 μL), blood plasma (50 μL), and urine (20 μL) were sampled for radioactivity and stored at − 20 °C.

HPLC Assay of Cyclic Imide 4. Bile (30−100 μL) from rats administered **4** (50 μmol/kg) was chromatographed at room temperature on a Symmetry 5 μm C-8 column (3.9 mm × 150 mm; Waters Corp, Milford, MS) using a gradient of acetonitrile (10−30% over 30 min, 30−70% over 5 min) in 10 mM ammonium acetate. The flow rate was 0.9 mL/min. The LC system consisted of two Jasco PU980 pumps (Jasco UK, Great Dunmow, Essex, UK) and a Jasco HG-980-30 mixing module. Eluted **4** (38 min) was identified by cochromatography with authentic compound and matching of electrospray mass spectra, and its absorbance measured at 254 nm with a Jasco UV-975 spectrophotometer. A linear (r^2 =

0.99) calibration graph (10 − 75 nmol) was constructed from an acetonitrile solution of authentic imide (1 mM). Peak areas of **4** were computed using MassLynx 3.5 integration software (Waters Corp, Manchester, UK). The intraassay coefficient of variation was 10% ($n = 6$).

LC-MS and Radiochromatographic Analyses. Aliquots (10−100 μL) of aqueous chemical reaction mixtures and solutions of synthetic compounds (1−2 mg/mL) were chromatographed on a Symmetry 5 μm C-8 column as described above unless otherwise indicated. Synthetic compounds were dissolved in acetonitrile except for glucuronides and the adducts of *N*α-acetyl L-lysine and *N*α-acetyl L-cysteine, which were dissolved in acetonitrile−1.0% aqueous acetic acid (2:1, v/v). Acyl glucuronide **3** was susceptible to transacylation by methanol at room temperature. Relative quantities of reactants and products were estimated from peak areas of absorbance at 254 nm. Radiolabeled compounds in bile (20−80 μL) and urine (100 μL) were eluted with a gradient of acetonitrile (20−50% over 30 min, 50−70% over 5 min) in formic acid (0.1%, v/v). The eluate was monitored at 254 nm. Eluate split-flow to the LC-MS interface was ca. 40 μL/min. A Quattro II mass spectrometer (Waters Corp, Manchester, UK) fitted with the standard coaxial electrospray source was operated using nitrogen as the nebulizing and drying gas. The interface temperature was 80 °C; the electrospray capillary voltage was 3.8 kV. Full scan spectra were acquired in the positive-ion mode between *m*/*z* 50−1050 over a scan duration of 5 s. Fragmentation of analyte ions was achieved at cone voltages between 25 and 50 V. Selected mass chromatogram peaks were integrated using MassLynx 3.5 software. Radiolabeled analytes were quantified with a Radiomatic A250 flow scintillation analyzer (Perkin-Elmer, Pangbourne, Berkshire, UK) as described previously.[48]

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Supporting Information Available: Microanalytical and high-resolution mass spectrometric data for **3**, **4**, **8**−**10**, **12**, **17a**, and **18a**, syntheses of **8**, **9**, and **11**, and spectroscopic and analytical data for **14** prepared by a published method. This material is available free of charge via the Internet at http://pubs.acs.org.

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