Discovery of a "True" Aspirin Prodrug

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Aspirin prodrugs formed by derivatization at the benzoic acid group are very difficult to obtain because the promoiety accelerates the rate of hydrolysis by plasma esterases at the neighboring acetyl group, generating salicylic acid derivatives. By tracing the hydrolysis pattern of the aspirin prodrug isosorbide-2,5-diaspirinate (ISDA) in human plasma solution, we were able to identify a metabolite, isosorbide-2-aspirinate-5-salicylate, that undergoes almost complete conversion to aspirin by human plasma butyrylcholinesterase, making it the most successful aspirin prodrug discovered to date.

Introduction

The design of an aspirin ester prodrug capable of enzyme-mediated activation is one of the classical problems in prodrug chemistry. Aspirin derivatives, such as esters and amides, are expected to be less toxic to the gastrointestinal tract (GIT)[a] following oral administration.[1] They usually have better aqueous stability than aspirin, making them potentially more suitable for a variety of pharmaceutical presentations.[2] There has accordingly been a lot interest in the area in the past both as a drug development opportunity and because the design of a successful prodrug represents a significant and interesting scientific conundrum.[3,4] Reported aspirin derivatives include aspirin anhydride,[5] benzodioxinone derivatives,[6] acylal derivatives,[7] *N*-(hydroxyalkyl) amides,[8] 2-formylphenyl derivatives,[9] straightforward alkyl and aryl esters,[10] triglycerides for lipase targeting,[11] acyloxyalkyl esters,[12] sulfinyl and sulfonyl esters,[13] phenylalanine amides,[14] amino acid derivatives,[15] indolediones as hypotoxic tissue targeting agents,[16] and dual release mechanism prodrugs.[17] Ideally, an aspirin prodrug should be stable under aqueous conditions and only undergo activation during the absorption and distribution process.[18]

The obstacle to aspirin prodrug development is that aspirin esters (**1**) are hydrolyzed rapidly by butyrylcholinesterase ([BuChE, EC3.1.1.8]) present in human plasma to the corresponding salicylate ester (**2**) and then, more slowly, to salicylic acid (**3**) [Figure 1a].[18] Aspirin (**4**) is comparatively stable in plasma, being a poor substrate for BuChE because of low affinity, a phenomenon observed for that enzyme with negatively charged substrates.[19] Neutral aspirin esters behave more like phenyl acetate (**5**), a "supersubstrate" for BuChE. For example, the half-life for the hydrolysis of phenylaspirinate (**6**) in 10% human plasma is 1.3 min, and it occurs exclusively at the acetate group. Aspirin under similar conditions undergoes hydrolysis at its acetate group, with a half-life of about 2 h.[18] As pointed out by Bundgaard and Nielsen, for a prodrug to release aspirin in plasma, the benzoic ester must undergo hydrolysis at a greater rate than at the acetyl group, whose hydrolysis is significantly accelerated by the presence of the benzoic ester. The implication is that a successful prodrug candidate in the plasma model requires a carrier group of even greater compatibility than the phenyl acetate or better still one which at the same time depresses the phenylacetate hydrolysis. The pseudo-choline glycolamide esters (Figure 1b, **7**) vindicated this strategy with hydrolysis, in the best case, to aspirin and the corresponding salicylate esters in a ratio of around 1:1.[18]

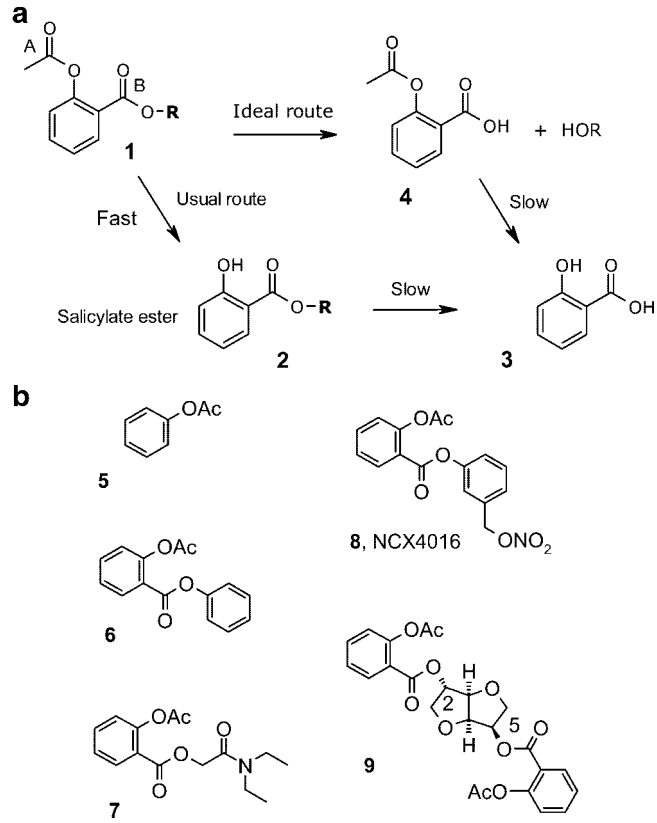

Figure 1. (a) Routes of hydrolysis of aspirin esters. (b) Phenyl acetate (**5**), some aspirin esters, and prodrugs.

Interest in the aspirin prodrug area has been renewed with the advent of the so-called nitro-aspirins and related compounds.[20] These are mutual prodrugs, in which aspirin is connected via an ester group to a nitric oxide releasing moiety, such as a nitrate ester, e.g., NCX4016 (**8**) and the aspirin diazenium diolates.[21,22] However, to exert both actions of its

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[a] Abbreviations: BNPP, bis-*p*-nitrophenylphosphate; BuChE, butyrylcholinesterase; BW254c51, 1:5-bis(4-allyl-dimethyl)ammoniumphenyl-pentan-3-one; DCC, dicyclohexylcarbodiimide; GIT, gastrointestinal tract; HPLC, high-performance liquid chromatography; ISDA, isosorbide-2,5-diaspirinate; EDTA, ethylenediaminetetraacetic acid; ISMN, isosorbide mononitrate; ISMNA, isosorbide mononitrate aspirinate; *iso*-OMPA, tetraisopropylpyrophosphoramide; PDA, photodiode array detector; TXB_2, thromboxane B_2.

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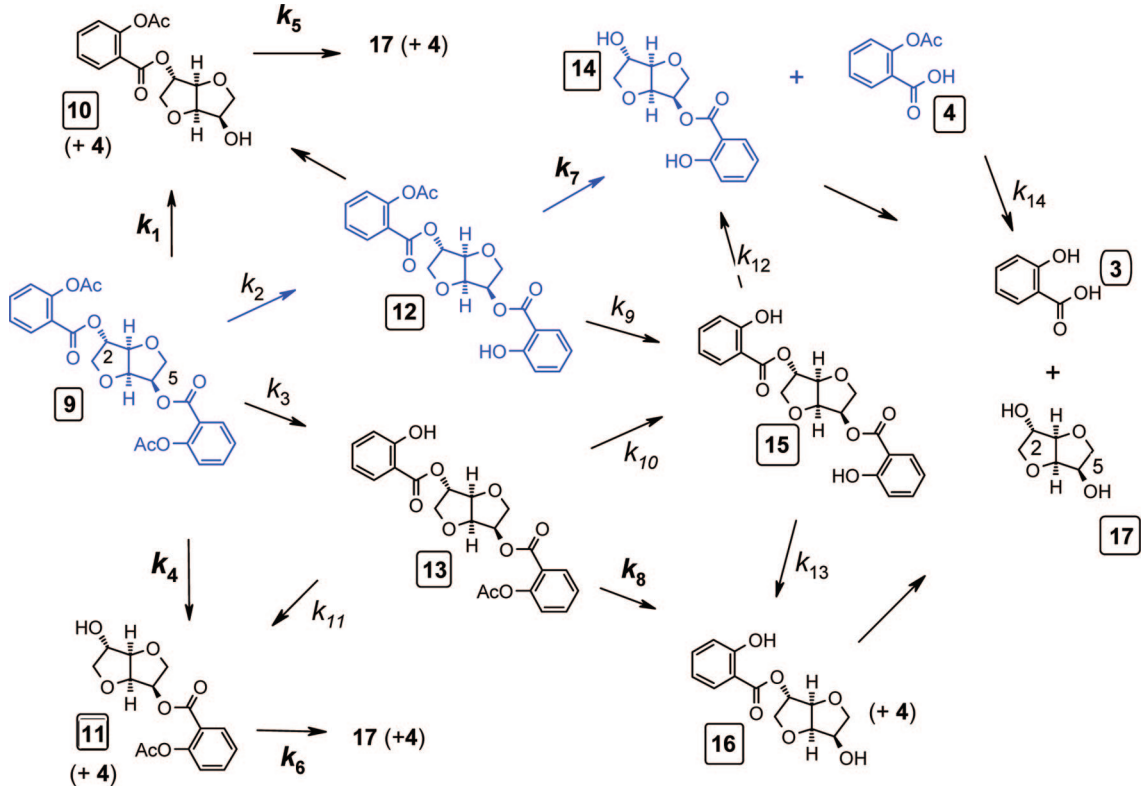
Scheme 1. ISDA Potential Hydrolysis Routes[a]



[a] All of the possible hydrolysis metabolites are illustrated, but some potential pathways are omitted for clarity; e.g., **16** may be generated from **10** and **14** from **11**. Processes leading directly to aspirin (**4**) are highlighted in bold. All processes lead ultimately to salicylic acid (**3**) and isosorbide (**17**).

component parts, a nitro-aspirin must be able to release aspirin. None of the compounds reported thus far in this category appear able to satisfy this requirement.[23]

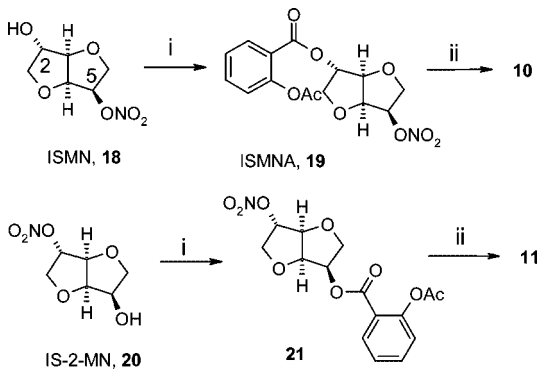
We reported some interesting characteristics of aspirinate esters of ISMN (ISMNA)[24] and then on the diaspirinate ester of isosorbide (ISDA, **9**, Figure 1). This latter diester produces significant amounts of aspirin when incubated in human plasma.[25] In a single oral dose study in dogs, ISDA administration caused sustained inhibition of *ex vivo* platelet function and TXB_2 production up to 24 h.[26] For an aspirin ester to produce aspirin *in vitro* and exhibit aspirin-like effects *ex vivo* is unusual and potentially valuable. The aim of the present study was to investigate the hydrolysis pathways of ISDA in human plasma and to explain its ability to produce aspirin. The potential metabolites of ISDA were synthesized, and their evolution and decay were monitored chromatographically following incubation in the presence of human plasma. We were surprised to discover that aspirin is produced from a hydrolysis product of ISDA along a pathway involving the first "true" aspirin prodrug. The compound behaves as an aspirin prodrug because of a highly specific and efficient interaction with human BuChE.

Results and Discussion

Approach toward Characterizing the Hydrolysis of ISDA. ISDA hydrolysis in human plasma solution is rapid ($t_{1/2}$ = 1.1 min in 30% plasma solution), leading to a complex mixture of aspirinate and salicylate metabolites as identified by HPLC/PDA (the salicylates have a characteristic UV λ_{max} at around 305 nm).[25] Around 60% aspirin is produced on the basis of starting ester concentration of the diester. ISDA has four ester bonds, one to each of the aspirin moieties (at the isosorbide 2 and 5 positions), as well as one acetyl group on each of the aspirin moieties. Hydrolysis of ISDA can occur through a network of parallel competing and consecutive pathways, with four initial routes characterized by the rate constants k_1, k_2, k_3, and k_4 (Scheme 1). Aspirin production could arise because of a direct hydrolysis on ISDA at either the isosorbide 2 or 5 positions, leading to primary monoaspirinate products **10** or **11** (k_1 and k_4 in Scheme 1), or because of hydrolysis on one of these monoaspirinates (k_5 and k_6 in Scheme 1), leading to isosorbide (**17**). Hydrolysis of the primary mixed salicylate−aspirinate compounds **12** or **13** along the k_7 and k_8 pathways would also lead to aspirin liberation. Altogether there are six possibilities for aspirin production from ISDA in the presence of plasma esterases. However, given the highly specific requirements for enzyme-mediated aspirin release from an aspirin ester, it seemed more likely that a smaller number of processes, possibly only one, leads to aspirin evolution from ISDA. We sought to characterize the distribution of ISDA metabolites in human plasma solution and to identify the productive pathway. Accordingly, the potential hydrolysis metabolites appearing in Scheme 1 were independently synthesized for use as standards to monitor hydrolysis progress and to evaluate their ability to generate aspirin separately.

Synthesis of ISDA Hydrolysis Products. Isosorbide mononitrate aspirinate (ISMNA,[24] **19**) was identified as a key intermediate in the synthesis of potential metabolites, and it was prepared from ISMN (**18**). To access the 2-monoaspirinate (**10**), the 5-nitrate was removed using H_2 in the presence of Pd/C in methanol/ethyl acetate (Scheme 2). The isomeric isosorbide-5-aspirinate (**11**) was prepared by a similar strategy (Scheme 3), employing isosorbide-2-mononitrate (**20**) instead.[27] The monosalicylates (**14** and **16**) were prepared by coupling of the appropriate mononitrates (**18** and **20**) to salicylic acid (DCC/DMAP) and removal of the nitrate group using Pd/C under an atmosphere of H_2. The disalicylate (**15**) and mixed 2/5-aspirinate-

Scheme 2. Synthesis of Isosorbide-2- and 5-Aspirinates **10** and **11**[a]



[a] (i) Acetylsalicoyl chloride, Et_3N, DCM, RT, 8 h; (ii) H_2, Pd/C, EtOAc/MeOH, RT, 8 h.

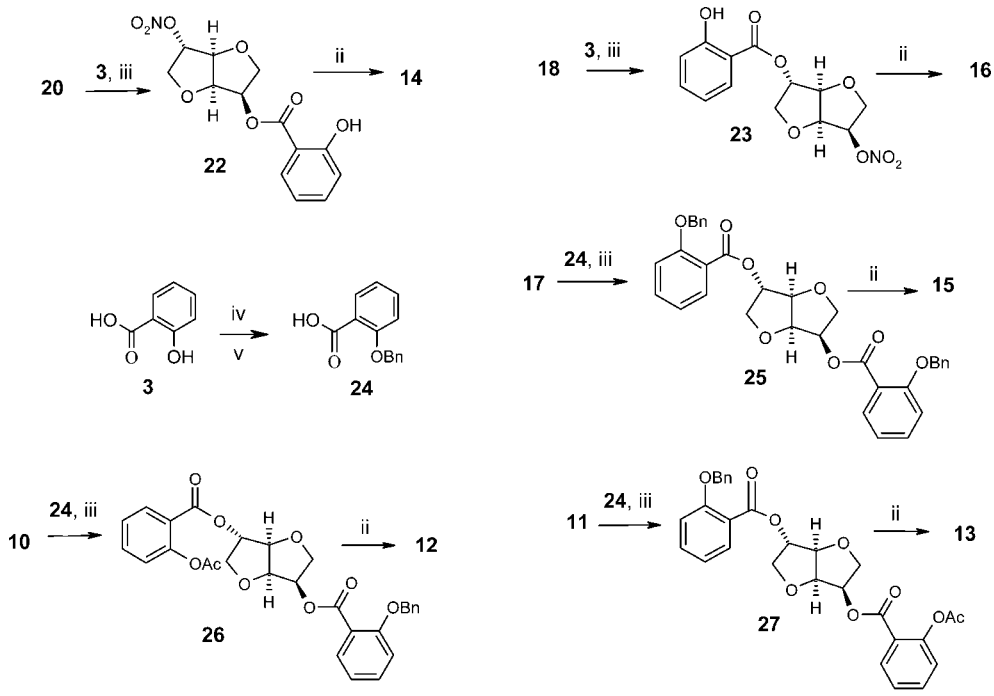
2/5-salicylates (**12** and **13**) could not be prepared by this directed acylation with salicylic acid because of competition between the salicylic acid-OH group and the isosorbide-OH group. Instead, salicylic acid was modified first by perbenzylation using benzylbromide (Scheme 3). The benzylester was removed by hydrolysis, leaving the phenolic benzylether intact. The disalicylate **15** was prepared by diesterification of isosorbide (**17**) with 2 equiv of **24** and removal of both benzyl groups, again by treatment under reductive conditions. Finally, the mixed 2-aspirinate-5-salicylate (**12**) and isomeric 5-aspirinate-2-salicylate (**13**) were obtained by esterification of the corresponding monoaspirinates (**10** and **11**) with **24** and removal of the benzyl groups as before. All of the potential metabolites were characterized appropriately.

Incubation Experiments with ISDA: Identification of Prodrug Metabolite. ISDA was incubated in human plasma solution in the range of 10−50% (pH 7.4, 37 °C), and the evolution and decay of its primary and secondary hydrolysis products monitored by HPLC using the synthetic external reference standards (Table 1). ISDA undergoes rapid hydrolysis at a high plasma concentration (50%) with apparently instantaneous evolution of aspirin. The apparent first-order rate constant (k_T) for the disappearance represents the combined rate constants for the primary k_1, k_2, k_3, and k_4 processes (Scheme 1). We were unable to detect any isosorbide-2-aspirinate (**10**) or the corresponding salicylate (**16**) at any time point, suggesting that the k_1 pathway does not play a significant role in the plasma-mediated hydrolysis of ISDA or in aspirin production. This interpretation, which was later vindicated, also ruled out the hypothetical k_5 pathway, which requires the availability of **10**. Hydrolysis of isosorbide-2-esters is very rapid, especially where the 5 position is substituted, and the failure to detect 2-aspirinate (**10**) or the corresponding salicylate ester (**16**) was not that surprising.

The complex pattern of the hydrolysis progress for ISDA in 10% human plasma solution is illustrated in the progress curve appearing in Figure 2. The principal initial hydrolysis products were the isosorbide-2/5-aspirinate-salicylate isomeric pair **12** and **13**, then the disalicylate **15**, the monosalicylate **14**, and later, salicylic acid. We were surprised to find that aspirin production from ISDA accelerated as the parent disappeared, with an apparent burst late in the decay profile. This pulsative behavior, which was reproducible in 10% plasma solution was not evident at higher plasma concentration (30%), where the disappearance of ISDA is extremely rapid (Figure 3). The appearance of aspirin in the 10% plasma solutions seemed to correlate with the consumption of the aspirinate−salicylate pair **12** and **13**, implying that one or both of these acts as an aspirin prodrug. A second curious feature of the progress curve was the predominance of the monosalicylate **14** over salicylic acid. Compound **14** can be generated from **11**, **12**, or **15**. However, if compound **14** was formed from **15**, an equimolar amount of salicylic acid would be generated, suggesting that it was formed from either **11** or **12**. Only small amounts of the 5-aspirinate (**11**) were detected (~10%) at several early time points, suggesting that it was not the source of **14**, although it remained possible that **11** was processed more rapidly than it formed. The salicylate−aspirinate pair **12** and **13** were consistently produced under a variety of conditions in a ratio of 70:30 independent of the plasma concentration (and therefore independent of the rate of disappearance of **9**). Because aspirin is a significant product following ISDA incubation in plasma, we hypothesized that the more significant salicylate−aspirinate metabolite, **12**, was acting as an aspirin prodrug. The substantial amount of **14** was consistent with this observation. To investigate this possibility, **12** and **13** were incubated separately in human plasma. Kinetic data for the disappearance of these and other potential aspirin-producing metabolites (**10**−**11**) are presented in Table 1. The disappearance profile in each case had the appearance of first-order kinetics, which is typically the case for Michaelis−Menten processes at low substrate concentration (and the rate constant relates to V_{max}/K_m). Compound **12** disappeared rapidly, with a half-life of <1 min in human plasma (50%), serum (50%) and whole blood, producing 70−90% aspirin based on the initial concentration, along with a corresponding amount of the monosalicylate "carrier" **14** (Figure 4). The disalicylate compound (**15**) was produced in small amounts (<15%), and it decayed rapidly afterward. The carrier (**14**) and disalicylate were hydrolyzed eventually to isosorbide and salicylic acid substances with well-characterized safety profiles and good tolerability. It is likely that this pattern would be followed *in vivo*.

Compound **12**, isosorbide-2-aspirinate-5-salicylate, is the most successful aspirin prodrug discovered to date in the important human plasma model. The isomeric 5-aspirinate-2-salicylate **13** did not produce aspirin at all, being exclusively hydrolyzed to isosorbide disalicylate (**15**) along the k_{10} pathway. The other prodrug candidates, the monoaspirinates **10** and**11**, were also unproductive, being rapidly hydrolyzed to the corresponding salicylates (a progress curve for the disappearance of **10** in human plasma solution is presented in Figure 5). The apparent Michaelis−Menten parameters in Table 1 were estimated by nonlinear regression of the disappearance data for each compound to the integrated form of the Michaelis−Menten equation.[28] The K_M^{app} values for the disappearance of these esters is difficult to interpret. In the case of **9**, for example, the K_M^{app} represents a mean value for three parallel processes involving three different productive presentations of the same compound at the BuChE active site. Equally, whereas the concentration of substrate decays below its K_M^{app} value, it generates an equimolar set of species that undergo processing with similar efficiency, so that active site occupancy remains constant over the initial phase of the reaction. Nevertheless, the K_M^{app} values provide us with a crude indication of the catalytic efficiency for the hydrolysis processes and the relative affinities of the substrate. The failure to detect any relationship between K_M^{app} or V_{max}^{app} values and aspirin production for compounds **9**−**13** illustrates that one kinetic process is only marginally preferred over the other even where aspirin production dominates.

Scheme 3. Synthesis of Salicylates **12**−**16**[a]



[a] (i) Acetylsalicoyl chloride, Et_3N, DCM, RT, 8 h; (ii) H_2, Pd/C, EtOAc/MeOH, RT, 8 h; (iii) DCC, DMAP, DCM, RT, 4 h; (iv) Cs_2CO_3, acetone, evacuation, and then BnBr; (v) NaOH, H_2O/MeOH, RT, 4 h.

Table 1. Kinetic Data for the Hydrolysis of ISDA (**9**) and Potential Aspirinate Metabolites **10**−**13** in Human Plasma Solution and for **12** in Other Relevant Media[a]

compound	$K_{m,app}$ (M, $\times 10^4$)	$V_{max,app}$ (M min^{-1})	k_{obs} (min^{-1})	$t_{1/2}$ (min)	aspirin yield (%)
9 (ISDA), 10% human plasma	1.59	0.3	0.20 ± 0.04	3.35	51 ± 10
9, 30% human plasma			0.59 ± 0.15	1.17	61 ± 4
9, 50% human plasma			1.2 ± 0.35	0.57	
10,10% human plasma	0.61	0.22	0.21 ± 0.04	3.25	3
11, 10% human plasma	1.11	0.25	0.15 ± 0.04	4.73	nd
13, 10% human plasma	0.91	0.23	0.16 ± 0.04	4.21	nd
12, 10% human plasma	0.81	0.17	0.17 ± 0.05	4.9	74 ± 8
12, 50% human plasma	0.69	0.36	1.39 ± 0.13	0.49	81 ± 11
12, 50% human serum	0.93	0.85	1.22 ± 0.29	0.56	73 ± 5
12, whole blood	1.41	1.49	0.79 ± 0.25	0.96	72 ± 13
12, 10% rat plasma	1.34	1.75	1.5 ± 0.56	0.47	nd

[a] All measurements were performed in triplicate. The k_{obs} range is the standard deviation for three independent measurements sometimes on different days or with different donor plasma, and they reflect biological variation in BuChE activity and specificity. The r^2 values for individual decay determinations were >0.995 in all cases.

Identification of Human BuChE as Responsible for Activation of Compound 12. There are a number of proteins in human plasma with esterase activity, including BuChE, paraoxonase (EC 3.1.8.1), trace amounts of acetylcholinesterase (EC 3.1.1.7), and albumin, which, although not highly efficient, is present at high concentrations (50−60 g/L).[29,30] Human plasma does not contain carboxylesterase.[31] When compound **12** was incubated in rat plasma solution (10%), the exclusive hydrolysis product was the disalicylate (**15**); there was no aspirin production (Table 1). Rat plasma esterase activity is largely due to carboxylesterases rather than cholinesterases.[31] ISDA had already been established as a substrate for human BuChE; therefore, it seemed likely that its aspirin-producing metabolite would be too. Compound **12** was nevertheless incubated in human plasma solution in the presence of esterase subtype inhibitors to confirm the identity of BuChE as the activating enzyme (Table 2). The presence of EDTA (arylesterase), BNPP (serine protease), or BW254c51 (0.1 μM, AChE) had little or no effect on aspirin production. There was a significant decrease in the rate of disappearance and aspirin production when the latter experiment was repeated with BW254c51 (100 μM), at which concentration it inhibits BuChE and AChE. Co-incubation with the selective BuChE inhibitor *iso*-OMPA (10 μM) and unselective cholinesterase inhibitor eserine (20 μM) caused a complete blockade of aspirin production and a significant drop in the hydrolysis rate. There was a significant drop in the hydrolysis rate and aspirin production following incubation in the presence of dibucaine in normal plasma but little difference in plasma from a donor previously classified as having the BuChE mutation conferring dibucaine resistance. Compound **12** was incubated in solution containing purified BuChE from human serum and in the presence of horse serum BuChE. Hydrolysis in the presence of the horse enzyme occurred at the acetyl and benzoic acid sites in a ratio of around 60:40. The purified human serum enzyme catalyzed hydrolysis almost exclusively toward aspirin, with only around 5% salicylate formation (Figure 6). The exceptionally high specificity of the purified BuChE suggests that there were competing nonproductive processes occurring in the plasma and serum experiments. Indeed, in the presence of the arylesterase inhibitors, there was

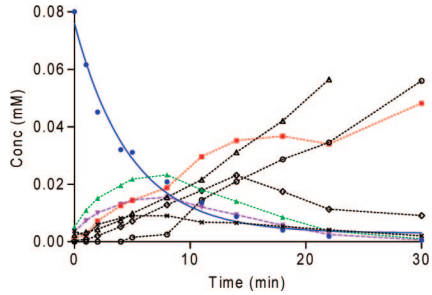


Figure 2. Progress curve for the disappearance of ISDA (**9**) (●) in 10% human plasma at 37 °C, showing the *in vitro* metabolites: aspirin (■), isosorbide-5-aspirinate (**11**) (×), isosorbide-2-aspirinate-5-salicylate **12** (▲), isosorbide-5-aspirinate-2-salicylate **13** (▼), salicylic acid, **3** (○), isosorbide-5-salicylate, **14**, (△), and isosorbide disalicylate, **15** (◇). The solid line for ISDA disappearance was calculated from exponential decay, using the rate constant appearing in Table 1. The data represent the average from three separate experiments using plasma from three different donors.

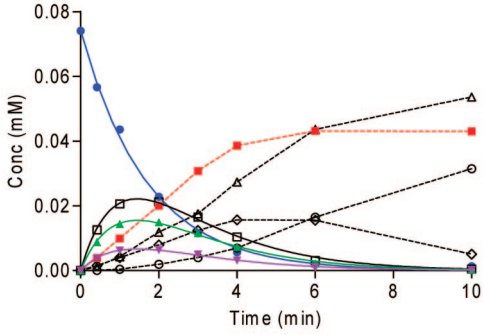


Figure 3. Progress curve for the disappearance of ISDA, **9**, (●) in 30% human plasma at 37 °C, showing the *in vitro* metabolites: aspirin (■), the pair **12** + **13** (□), isosorbide-2-aspirinate-5-salicylate, **12** (▲), isosorbide-5-aspirinate-2-salicylate, **13** (▼), salicylic acid, **3** (○), isosorbide-5-salicylate, **14**, (△), and isosorbide disalicylate, **15** (◇). Solid lines were constructed using rate constants appearing in Table 3 and eqs 1−3.

marginally greater aspirin release, suggesting that an arylesterase, probably paraoxonase, contributes to nonproductive hydrolysis in human plasma. The hydrolysis pathways of **12** were similar in human plasma, serum, and whole blood, with the esterase activity of the latter being substantially a result of the presence of BuChE. It is hard to see how **12** could be further optimized for aspirin release in human plasma using BuChE as the vector because BuChE is almost completely specific for the benzoic ester of **12**. On the other hand, designs that target other plasma esterases, such as paraoxonase, must still overcome the high efficiency with which acetyl group detachment is catalyzed by BuChE.

Is ISDA (9) Intrinsically an Aspirin Prodrug? Compound **12** acts as an aspirin prodrug because its interaction with BuChE overrides the normal preference of the enzyme for the phenylacetate group. This interaction is connected with a specific set of structural attributes of the prodrug; for example, the isomeric 2-salicylate-5-aspirinate (**13**) is not an aspirin prodrug. We were also aware that isosorbide-2-aspirinate (**10**) (unsubstituted at the 5 position) was not an aspirin prodrug being hydrolyzed in plasma solution along the salicylate pathway (Figure 5). This indicated to us that the 5-salicylate ester of **12** is critical for productive binding, leading to aspirin evolution. ISDA is hydrolyzed first at the acetyl group of the 5-aspirinate, producing **12**, which acts as a prodrug in human plasma. Does ISDA itself

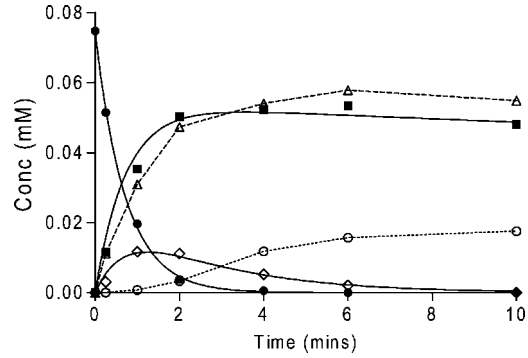


Figure 4. Progress curve for the disappearance of **12** (●) in 50% human plasma buffered at pH 7.4 (37 °C), showing the *in vitro* metabolites: aspirin, **4** (■), isosorbide-5-salicylate, **14** (△), salicylic acid, **3** (○), and isosorbide disalicylate, **15** (◇). Note the stability of **14** relative to **12**. Solid lines were constructed using the appropriate rate constants appearing in Table 3 and eqs 4−6.

act as a prodrug, without having to generate **12**? This was not a straightforward question because **12** is generated from **9** and consumed rapidly, producing aspirin. As already stated, small amounts (<10%) of the 5-monoaspirinate **11** were found at initial time points in 10% plasma solution, indicating some direct hydrolysis of **9** to aspirin. The only other way that **11** could be generated is from the mixed salicylate **13**, but when **13** was incubated separately in human plasma solution, it did not produce **11**, indicating that, in plasma solution, **11** is produced directly from ISDA, with an equimolar amount of aspirin (<10%). Another way of investigating the production of aspirin from ISDA would be to determine how much of its hydrolysis could be accounted for by alternative processes to salicylate production.

ISDA decays through three pathways, k_2, k_3, and k_4, and therefore

$$-\mathrm{d}[\mathbf{9}]/\mathrm{d}t = k_T[\mathbf{9}] = k_2[\mathbf{9}] + k_3[\mathbf{9}] + k_4[\mathbf{9}] \quad (1)$$

Because **12** decays through the k_7 and k_9 pathways, leading to aspirin and disalicylate (**15**)

$$\mathrm{d}[\mathbf{12}]/\mathrm{d}t = k_2[\mathbf{9}] - k_7[\mathbf{12}] - k_9[\mathbf{12}] \quad (2)$$

Similarly, **13** decays exclusively through the k_{10} pathway and therefore

$$\mathrm{d}[\mathbf{13}]/\mathrm{d}t = k_3[\mathbf{9}] - k_{10}[\mathbf{13}] \quad (3)$$

By estimating k_2 and k_3 from nonlinear regression to integrated forms of eqs 2 and 3, it was possible to estimate k_4, the rate constant for aspirin production directly from ISDA. In calculating these kinetic parameters, k_T was estimated from the disappearance of **9** in 30% plasma solution (Table 3). Initial values for the disappearance of esters **12** and **13** were estimated from the rate of hydrolysis independent of **9**. The difference between the sum of the rate constants for the appearance of **12** and **13** and the disappearance rate for ISDA yielded a small value for k_4, indicating that around 7% of **9** undergoes hydrolysis directly to aspirin (Table 3). ISDA is an intrinsic aspirin prodrug in plasma but a poor one. It probably fits the enzyme quite well considering its similarity to **12**, but it bears two acetates and therefore has two competing processes rather than one, as is the case for **12**.

Interpretation of hydrolysis data for **12** is kinetically simpler because it has one less ester group than **9** and its principal product, aspirin, is not consumed as rapidly (Scheme 4). In addition, there was no detectable hydrolysis at the 5-ester over

Table 2. Data for the Hydrolysis of **12** in 30% Human Plasma (pH 7.4, 37 °C) in the Presence of Esterase Subtype Inhibitors[a]

inhibitor	concentration	target enzyme	k_{obs} (min^{-1})	$t_{1/2}$ (min)	% aspirin
EDTA[32]	2 mM	arylesterase	0.49 ± 0.08	1.41	85
BNPP[33]	10 μM	carboxylesterase	0.73 ± 0.006	0.95	84
eserine[34]	20 μM	cholinesterase	0.12 ± 0.001	5.8	not detected
BW284c51[35]	100 μM	AChE	0.18 ± 0.001	3.9	30
	0.1 μM		0.66 ± 0.064	1.1	74
dibucaine[36]	20 μM	BuChE subtype	0.15 ± 0.013	4.6	6
			0.35 ± 0.07	2.0[b]	69[b]
iso-OMPA[37]	10 μM	BuChE	0.098± 0.003	7.04	not detected

[a] Average of three determinations ± standard deviation (SD). [b] Plasma from a donor with dubucaine-insensitive BuChE.

Table 3. Rate Constants for the Hydrolysis of **9** and **12** in Human Plasma (pH 7.4, 37°C)[a]

compound	k_{obs}	k_2	k_3	k_4	$k_2+ k_3/k_{obs}$	k_7'	k_9'[b]	k_7'/k_{obs}
9[c]	0.591± 0.05	0.39 ± 0.02	0.16 ±0.02	0.028 ± 0.01	0.93			
12[d]	1.39 ±0.11	nr	nr	nr		1.0± 0.06	0.36 ± 0.06	0.72

[a] Data are from a single representative progress curve. $r^2 > 0.999$. [b] $r^2 > 0.95$. [c] A 30% plasma solution. [d] A 50% plasma solution.

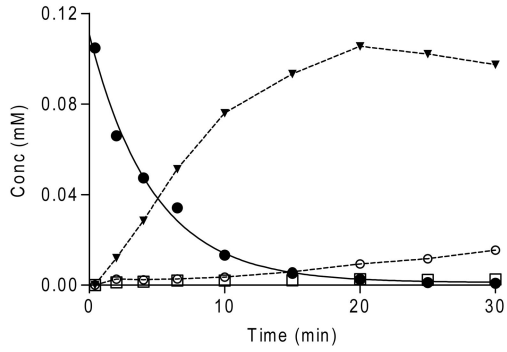


Figure 5. Progress curve for the disappearance of isosorbide-2-aspirinate (**10**) (●) in 10% human plasma solution at pH 7.4 (37 °C), showing the *in vitro* metabolites: aspirin (□), isosorbide-2-salicylate, **16** (▼), and salicylic acid, **3** (○). The solid line was constructed using rate constants appearing in Table 1.

Table 4. IC_{50} Values for Inhibition of Platelet Aggregation in Whole Blood Stimulated by Arachidonic Acid (0.5 mM), $n = 3$

compound	IC_{50}, 95% CL (μM)
9	34.1 (28.2−44.3)
12	17.3 (15.9−18.8)
aspirin	25.5 (20.1−31.7)

the time course of the experiment; it does not contribute to the disappearance of **12**. The k_{obs} for the consumption of **12** (k_T') corresponds to the sum of the rate constants for deacetylation to **15**, k_9' and the larger rate constant for hydrolysis to aspirin, k_7' (where k_T', k_7', and k_9' are rate constants determined for the hydrolysis of **12** independent of **9**).

$$-\mathrm{d}[\mathbf{12}]/\mathrm{d}t = k_T'[\mathbf{12}] = k_9'[\mathbf{12}] + k_7'[\mathbf{12}] \quad (4)$$

Similarly, the disappearance of the disalicylate (**15**), following incubation of **12**, occurs along the k_{12}' and to a small extent the k_{13}' pathway

$$\mathrm{d}[\mathbf{15}]/\mathrm{d}t = k_9'[\mathbf{12}] - k_{12}'[\mathbf{15}] - k_{13}'[\mathbf{15}] \quad (5)$$

Aspirin evolution and decay are through the k_7 pathway and the slow decay to salicylic acid, k_{14}

$$\mathrm{d}[\mathbf{4}]/\mathrm{d}t = k_7'[\mathbf{12}] - k_{14}'[\mathbf{4}] \quad (6)$$

The value for k_7' obtained in this way was in good agreement with the product of k_T' and the maximum mole percent of aspirin extrapolated from the progress curve for **12** (Figure 4). Meanwhile, the k_7' value accounts for 72% of the total rate

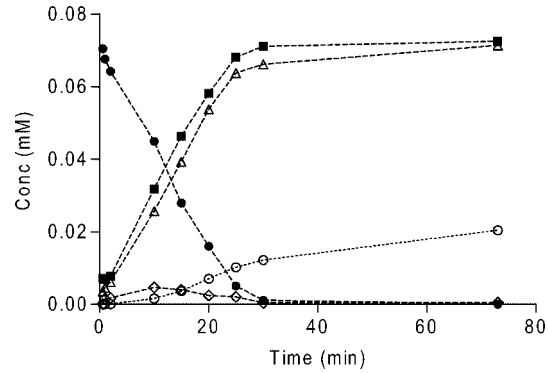
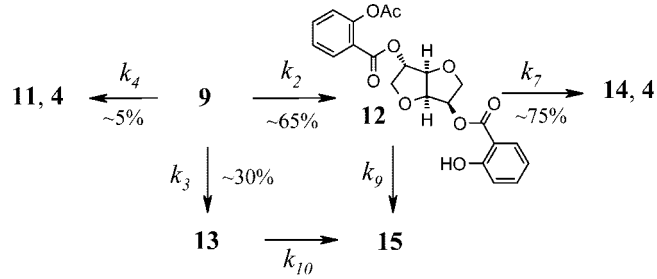


Figure 6. Progress curve for the disappearance of **12** (●) in the presence of BuChE purified from human serum buffered at pH 7.4 (37 °C), showing the *in vitro* metabolites: aspirin (■), isosorbide-5-salicylate, **14** (△), salicylic acid, **3** (○), and isosorbide disalicylate, **15** (◇). The data are for one representative run. The ratio of aspirin/disalicylate (**15**) at C_{max} for each was 95:5.

Scheme 4. Actual Hydrolysis Routes of ISDA[a]



[a] The 5-acetyl group is removed first (65%), generating an effective prodrug (**12**). The other primary route (k_3) involving 2-acetyl group hydrolysis does not lead to aspirin generation because **13** is not an aspirin prodrug.

constant for **12** disappearance, in accordance with the estimate from extrapolation of maximum aspirin during the disappearance of **12**. The data appearing in Scheme 4 is based on the assumption that the disappearance kinetics for **12** are similar in the presence of ISDA.

Platelet Aggregation Studies. One of the hallmark pharmacological effects of aspirin is its ability to interfere with platelet function through acetylation of platelet COX-1. Compound **12**, aspirin, and ISDA (**9**) were evaluated as inhibitors of platelet aggregation stimulated by arachidonic acid (0.5 mM) using the whole blood impedance method.[38] Compound **12** was significantly more potent than ISDA in accordance with its enhanced

ability to generate aspirin (Table 4). Puzzling, **12** was also marginally (but significantly) more potent than aspirin. Compound **10**, which did not release aspirin in the plasma hydrolysis experiment was not active in this assay (<30% inhibition at 100 μM), indicating that platelet inhibition by **12** and ISDA (**9**) is due to aspirin release.

Conclusions

Compound **12** is the first "true" aspirin prodrug in BuChE solutions, and it produces more aspirin in human plasma than any other prodrug discovered to date. It does so because of an unusually favorable interaction with BuChE in human plasma. These observations remind us that seemingly insurmountable esterase preferences can be overcome with appropriate substrate complementarity. A pseudo-sugar is probably the last place one would start in pursuit of a cholinesterase substrate type; however, BuChE does not have an established endogenous substrate or ligand, and the esters reported here are more successful substrates than the ostensibly more promising pseudo-choline esters reported by Nielsen and Bundgaard.[18] Compound **12** will be tested in appropriate preclinical models of platelet inhibition and then gastric toxicity. One of the challenges will be to develop and validate an animal model that mimics the ability of human blood to convert **12** to aspirin (which excludes, for example, the rat). Without aspirin generation, an ester can be expected to be safe but not efficacious.

Experimental Section

Chemistry. Infrared (IR) spectra were obtained using a Perkin-Elmer 205 FT Infrared Paragon 1000 spectrometer. Band positions are given in cm^{-1}. Solid samples were obtained by KBr discs, and oils were analyzed as neat films on NaCl plates. ^1H and ^{13}C spectra were recorded at 27 °C on a Brucker DPX 400 MHz FT NMR spectrometer (400.13 MHz, ^1H; 100.61 MHz, ^{13}C) or a Brucker AV600 (600.13 MHz, ^1H; 150.6 MHz, ^{13}C) in either $CDCl_3$ or $(CD_3)_2CO$ with TMS as an internal standard. In $CDCl_3$, ^1H spectra were assigned relative to the TMS peak at 0.0 ppm and ^{13}C spectra were assigned relative to the middle $CDCl_3$ triplet at 77.00 ppm. In $(CD_3)_2CO$, ^1H spectra were assigned relative to the $(CD_3)_2CO$ peak at 2.05 ppm and ^{13}C spectra were assigned relative to the $(CD_3)_2CO$ at 29.5 ppm. Coupling constants were reported in hertz. High-resolution mass spectrometry (HRMS) was performed using a micromass mass spectrophotometer with electrospray ionization at the School of Chemistry, Trinity College Dublin, Ireland. Elemental analyses were performed at the Microanalytical Laboratory, Department of Chemistry, University College Dublin, Ireland. Flash chromatography was performed on Merck Kieselgel (particle size of 60 mm). Thin-layer chromatography (TLC) was performed on silica gel Merck F-254 plates. Compounds were visually detected by UV absorbance at 254 nm. ISMN was obtained as a gift from Shwartz Pharma, Shannon Co Clare. Compound **24** was obtained according to ref 39.

Isosorbide-2-aspirinate-5-salicylate (12). Compound **24** (1.6 mmol) was dissolved in dry DCM (20 mL) and stirred. Isosorbide-2-aspirinate **10** (1.6 mmol) and DMAP (0.1 equiv) were added. The flask was cooled to 0 °C, and DCC (1.6 mmol) was added. Stirring was continued for 5 min, and the temperature was allowed to come to room temperature, where it was stirred overnight. The reaction was filtered, and the filtrate was washed with 0.1 M HCl, 5% $NaHCO_3$, and water, dried over sodium sulfate and evaporated to an oil. This was purified by column chromatography hexane/ethyl acetate (2:1) to give a white product (R_f = 0.4, 228 mg). This was dissolved in methanol/ethyl acetate (1:1). Pd/C was added, and the reaction was stirred under hydrogen overnight. The reaction was filtered and concentrated. Oil was purified by column chromatography using hexane/ethyl acetate (1:1) to yield a white solid. mp 82−84 °C. LRMS: requires, 451.0984 (M^+ + 23); found, 451.0971 (M^+ + 23). ^1H NMR ($CDCl_3$) δ: 2.37 (1H, s), 4.10 (4H, m), 4.64 (1H, d), 5.04 (1H, t), 5.45 (2H, m), 6.92 (1H, t), 7.02 (1H, d), 7.12 (1H, d), 7.32 (1H, t), 7.49 (1H, m), 7.58 (1H, m), 7.89 (1H, dd, J = 1.5 and 1.5 Hz), 8.01 (1H, dd, J = 1.5 and 1.5 Hz). Anal. Calcd ($C_{22}H_{20}O_9$): C, H.

Isosorbide-2-salicylate-5-aspirinate (13). A solution of isosorbide-5-aspirinate-2-benzyloxy benzoate (**27**) (0.13 g, 0.25 mmol) in a mixture of methanol and ethyl acetate (5 mL, 3:1) was stirred overnight over palladium on charcoal under an atmosphere of hydrogen. The reaction mixture was filtered through a bed of silica, and the filtrate was removed *in vacuo* to yield 0.05 g of crude product. Purification by column chromatography using hexane and ethyl acetate (2:1) as the eluant afforded 0.02 g of product as a colorless crystalline material (18.69%). mp 84−86 °C. HRMS [M + Na]$^+$: requires, 451.1005; found, 451.1006. ^1H NMR ($CDCl_3$) δ: 2.36 (3H, s), 4.03 (4H, m), 4.67 (1H, d), 5.02 (1H, t), 5.38 (1H, m), 5.49 (1H, d), 6.87 (1H, t), 6.98 (1H, d), 7.12 (1H, d), 7.34 (1H, t), 7.47 (1H, m), 7.58 (1H, m), 7.83 (1H, dd, J = 1.5 and 1.5 Hz), 8.08 (1H, dd, J = 2.0 and 1.5 Hz), 10.50 (1H, s). Anal. Calcd ($C_{22}H_{20}O_9$): C, H.

Hydrolysis and Enzyme Studies. High-performance liquid chromatography (HPLC) was performed using a system consisting of a Waters 600E pump and controller, a Waters 717 autosampler, and a Waters (2)996 PDA, all controlled by Millennium or Alliance Chromatography Manager. A Waters Spherisorb (4 μm) C18 3.9 × 250 mm column was used for the plasma and enzyme study samples. The enzyme and plasma study samples were analyzed using a gradient method, employing a mobile phase consisting of pH 3.19 phosphate buffer/acetonitrile at 90:10, grading to 10:90 over the first 10 min, then back to 65:35 to 12 min, and grading to 90:10 to 17 min, at which it was held to 30 min. The eluent in both methods was monitored at 230 nm, and peak identity and homogeneity were confirmed by PDA analysis. Quantitation was performed by a comparison of peak areas with external standards run under the same conditions at about the same concentration. The order of elution in this system was salicylic acid (**3**), aspirin (**4**), isosorbide-2-aspirinate (**10**), isosorbide-5-aspirinate (**11**), isosorbide-2-salicylate (**16**), isosorbide-5-salicylate (**14**), isosorbide-2/5-aspirinate-2/5-salicylate (**12/13**), and isosorbide-disalicylate (**15**). The flow rate was 1 mL/min. ISDA incubation samples were reanalysed using an alternative isocratic method, with a mobile phase consisting of 60% phosphate buffer (pH 2.4)/40% acetonitrile. This permitted the separation of **12** and **13**. Both methods (gradient and isocratic) were validated for linearity, precision (repeatability), specificity, and sensitivity in accordance with ICH guidelines on analytical validation Q2A and Q2B. A linear response was observed for each analyte (r > 0.999) in the range of 1−100 μg/mL. The RSD on multiple injection of each analyte at 10 and 100 μg/mL was <0.75%. The limit of quantitation for the relevant analytes in the gradient method was 1 μg/mL. The limit of quantitation for aspirin and salicylic acid in the isocratic method used in the aqueous hydrolysis study samples was 5 μg/mL, while the prodrug **12** was quantifiable at 0.5 μg/mL using this method.

For the hydrolysis experiments, pooled plasma or serum solutions (4 mL) were prepared by centrifugation of citrated human venous blood and dilution with phosphate buffer at pH 7.4, as appropriate. Whole blood was used undiluted. The test compounds in acetonitrile (100 μL) were incubated in 4 mL of the preheated solution (37 ± 0.5 °C) at a concentration of 5 × 10^{-5} M, and 250 μL aliquots were withdrawn at appropriate intervals. Samples were transferred to 1.5 mL Eppendorf tubes containing 500 μL of a 2% $ZnSO_4 \cdot 7H_2O$ in MeCN/H_2O (1:1) solution, vortexed, and then centrifuged for 4 min at 10 000 rpm. Aliquots (20 μL) of the supernatant were analyzed by HPLC. Hydrolysis was monitored until consumption of the parent ester. The hydrolysis experiments were repeated in the presence of the following inhibitors, which were incubated in the buffered plasma solution for 5 min before addition of the ester solution: EDTA (2 mM), BNPP (10 μM), eserine (20 μM), BW254c51 (0.1−100 μM), dibucaine (20 μM), *iso*-OMPA (10 μM). The samples were then processed as above. All experiments were performed in triplicate. Cholinesterase activity of plasma, serum, and whole blood was evaluated using the Ellman approach, with

butyryl thiocholine as the substrate. Values were typically between 2200 and 4000 nmol (mL of plasma)$^{-1}$ min^{-1}. The hydrolysis of **12** was evaluated in the presence of purified horse serum BuChE (Sigma) at a concentration of 0.1 mg/mL (1000 units/mg of protein) in phosphate buffer (pH 7.4) and in the presence of purified human serum BuChE at 0.1 mg/mL (14 units/mg of protein) (Sigma). The activity of these preparations was confirmed using the Ellman assay, with butyrylthiocholine as the substrate.[40]

The parameters K_M^{app} (Michaelis constant) and V_{max}^{app} (maximum rate of substrate consumption) for the hydrolysis of **12** were estimated by fitting depletion data to the integrated form of the Michaelis−Menten equation[28] by multiple nonlinear least-squares regression using Scientist, MicroMath (Salt Lake City, UT)

$$V_{max}t = S_o - S + K_M \ln(S_o/S) \quad (7)$$

Rate constants for the hydrolysis of esters were obtained by nonlinear regression of the concentration of remaining ester against time to exponential decay. Half-lives were calculated from $t_{1/2} = 0.693/k_{obs}$. Integrated forms of eqs 1−6 were used to determine the rate constants appearing in Table 3 and to generate the solid lines appearing in Figure 3 for ISDA hydrolysis[41]

$$[\mathbf{12}] = \frac{k_2[\mathbf{9}]_0}{k_T - (k_7 + k_9)}(e^{-(k_7+k_9)t} - e^{-k_T t}) \quad (8)$$

$$[\mathbf{13}] = \frac{k_3[\mathbf{9}]_0}{k_T - k_{10}}(e^{-k_{10}t} - e^{-k_T t}) \quad (9)$$

The following were used to generate the solid lines in Figure 4, for hydrolysis of **12**, separately:

$$[\mathbf{15}] = \frac{k_9'[\mathbf{12}]_0}{k_T' - (k_{12}' + k_{13}')}(e^{-(k_{12}'+k_{13}')t} - e^{-(k_T' t)}) \quad (10)$$

$$[\mathbf{4}] = \frac{k_7'[\mathbf{12}]_0}{k_T' - k_{14}'}(e^{-k_{14}'t} - e^{-k_T' t}) \quad (11)$$

Nonlinear regression to eqs 8−11 was performed using GraphPad Prism5 (La Jolla, CA).

Whole Blood Aggregation. A 500 μL aliquot of whole blood was mixed with 500 μL of physiological saline and allowed to incubate at 37 °C for 10 min in the incubation well of a Chrono-Log Whole Blood Aggregometer model 591/592. Aggregation was initiated with arachidonic acid (0.5 mM), and impedance was monitored over 6 min. During inhibition studies, the diluted blood sample was pre-incubated with appropriate concentrations of inhibitor at 37 °C for 10 min, with stirring before initiating aggregation. Test compounds (10 μL) were introduced in DMSO (a DMSO level shown to have no effect on platelet function). Inhibition of aggregation was monitored in the range of 5−100 μM, and results were analyzed by nonlinear regression using GraphPad Prism5.

Supporting Information Available: Characterization and purity data for compounds **10**, **11**, **14**, **15**, **16**, **20**, **21**, **23**, and **25** and platelet aggregation data for **9**, **12**, and aspirin. This material is available free of charge via the Internet at http://pubs.acs.org.

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