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Development of a fluorous trapping reagent for rapid detection of electrophilic reactive metabolites†

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A cysteine-based fluorous trapping reagent, Rf_8CYS, was developed. Rf_8CYS formed adducts with soft and hard electrophilic reactive metabolites. These fluorous-tagged adducts were purified *via* both fluorous solid-phase extraction and the direct injection method. The highly sensitive mass spectrometric detection of an unprecedented adduct of the ticlopidine metabolite was realized.

Reactive metabolites (RMs), which are formed from drug metabolism, have been associated with drug toxicity and idiosyncratic adverse drug reactions. Hence, the structures of the corresponding RMs must be determined at an early stage of drug development and optimized to reduce the aforementioned risks.[1–5] Owing to the unstable nature of RMs, a trapping assay is typically used for their detection and structural identification.[6–8] In a typical trapping assay, a nucleophilic trapping reagent is used to form a stable adduct with an electrophilic RM in liver microsomes. These adducts are detected using liquid chromatography/mass spectrometry (LC/MS). The reactivity of the RMs and trapping reagents follows the principle of hard and soft acids and bases. Soft nucleophilic groups, such as thiols, are more likely to react with soft RMs, such as quinones and epoxides, whereas hard nucleophilic groups, such as amines, are more likely to react with hard RMs such as aldehydes.[5]

Glutathione (GSH) and its derivatives are widely used as soft trapping reagents to capture soft nucleophilic RMs with thiol groups.[9–18] Methoxyamine and semi-carbazide are known trapping reagents for hard RMs.[19,20] γ-Glutamylcysteinlysine, which possesses both SH and NH_2 groups, serves as a trapping reagent for both soft and hard RMs.[21] Cysteine and its derivatives are also suitable for both types of RMs.[22–27] The SH and NH_2 groups in cysteine are connected by two carbons, and this molecular chain can effectively capture an aldehyde as a thiazolidine. However, the aldehyde is captured as an imine, which is less stable if only an amine is used as a trapping reagent.

In addition to their reactivity, trapping reagents must have excellent detection sensitivity. Under the conditions of the trapping assay, biological matrices derived from liver microsomes cause a matrix effect, which lowers MS sensitivity and increases background noise. This can complicate the detection and structural determination of adducts using LC/MS. Consequently, radioisotopes[22,24,27] or fluorescent functional groups[25,26] have been incorporated into cysteine or its derivatives to lessen the impact of biological matrices on the trapping assay and facilitate the highly sensitive detection of the RM adducts. However, these methods require the detection of radiation and fluorescence.

Perfluoroalkyl (fluorous) compounds can be easily separated from non-fluorous compounds based on the specific affinity between them.[28,29] Accordingly, fluorous tags have been used for the purification and analysis of various endogenous biomolecules.[30–38] In 2020, Hayama *et al.* generated GSH adducts from biological matrices *via* fluorous derivatization.[12] In their method, GSH adducts were subjected to a trapping assay and then derivatized with a light fluorous tag. The compounds were separated using LC/MS equipped with a fluorous silica gel column. Notably, fluorous-derivatized compounds exhibit much higher ionization efficiencies than those of non-derivatized compounds. High ionization efficiency is advantageous for MS detection. Therefore, the fluorous derivatization method enhances both sample purity and ionization efficiency, enabling the sensitive detection of RMs. However, the additional derivatization step requires more time and effort, and the RM adducts could be converted into other compounds during the derivatization reactions. To resolve these issues, in this study, we developed a novel fluorous-trapping reagent that enables effective identification of RMs without a post-derivatization step.

The trapping reagent, Rf_8CYS (**1**), comprised cysteine as the reactive group because of its ability to capture a wide range of electrophiles (Fig. 1) and a perfluorooctyl group with 17 fluorine

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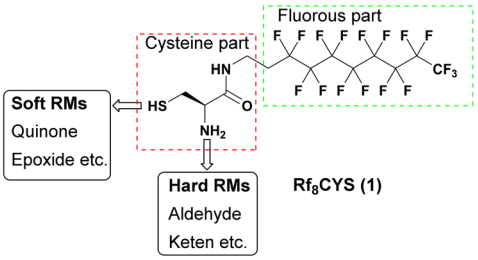


Fig. 1 Schematic of the design of our fluorous trapping reagent, Rf_8CYS, and its chemical structure.

atoms as the fluorous tag. This fluorous group was selected because (1) at least nine fluorine atoms are required for separating the fluorous tag using a fluorous silica gel column; and (2) the water solubility of the fluorous-derivatized compound increases with an decreasing number of fluorine atoms.[39] To prevent a decrease in the reactivity of cysteine owing to the strong electron-withdrawing inductive effect of the fluorous group, a two-carbon methylene spacer was introduced between the two parts of the trapping reagent.

Rf_8CYS and its non-fluorous derivative **2** (control compound) were synthesized from amine- and thiol-protected cysteine, respectively (Schemes S1 and S2†).

First, the MS sensitivities of the fluorous and non-fluorous trapping reagents were compared (Fig. S1†). At a concentration of 1.5 mmol L^{-1}, the signal-to-noise (S/N) ratio of Rf_8CYS was more than 10 times higher than that of **2**. Moreover, Rf_8CYS was detected with an S/N ratio of 7.8 even at a concentration of 0.015 mmol L^{-1}, whereas the detection limit of **2** was higher than 0.15 mmol L^{-1}. These results demonstrate that fluorous derivatization significantly improves MS sensitivity.

Despite having bulky, electron-withdrawing, and fluorophilic substituents, Rf_8CYS was sufficiently reactive toward RMs. To investigate the impact of fluorous groups on the reactivity of the metabolite, the efficiencies of Rf_8CYS (**1**) and **2** in detecting benzaldehyde were determined and compared (Fig. 2, S2, and S3†). The reactions were conducted in phosphate buffer (PBS, pH 7.4) with 1% MeOH because organic solvent concentrations exceeding 1% are not recommended to prevent the inhibition of hepatic microsomal metabolism in the trapping assay. Both reactions proceeded similarly to afford thiazolidine adducts **3** and **4**. However, the peak intensity of Rf_8CYS adduct **3** was 10 times stronger than that of **4**. These results suggest that the fluorous group did not affect the reactivity. The structure of adduct **3** was determined by comparing its retention time with that of an adduct synthesized using an alternative method. The same reaction was performed with cysteine. However, the expected adduct was not detected under the same conditions, owing to the low MS sensitivity of the cysteine adduct.

Subsequently, the applicability of Rf_8CYS to both hard and soft RMs was studied using model compounds (Table 1 and Fig. S4–S8†). Rf_8CYS reacted with electron-rich methoxybenzaldehyde, electron-deficient chlorobenzaldehyde, and alkyl aldehydes to form thiazolidines **5–7**. The reactions proceeded smoothly with the soft electrophiles (epoxide and *p*-benzoquinone). In addition to the expected adducts **8** and **10**, the unstable adduct **9**, which is an intermediate of **10**, was identified.

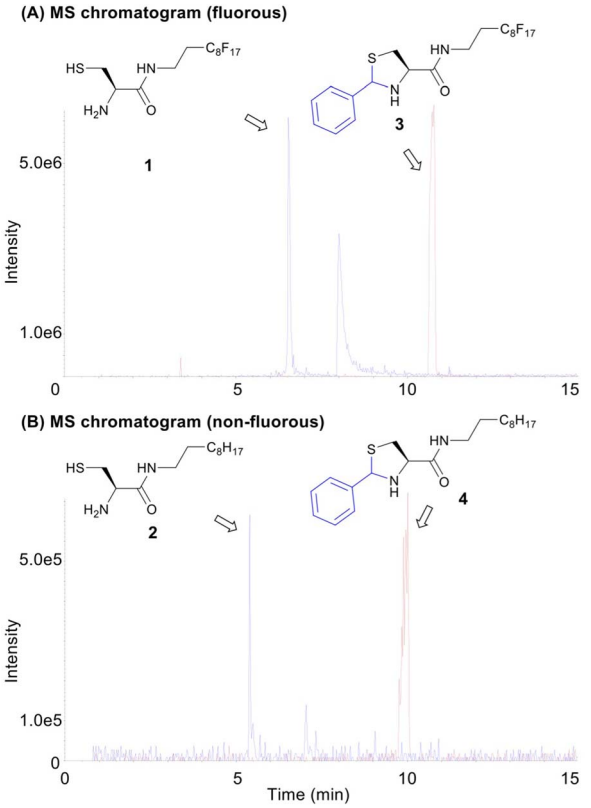


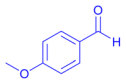
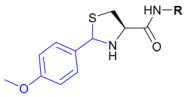
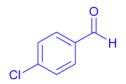
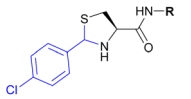
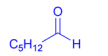
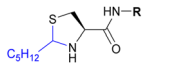
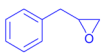
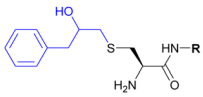
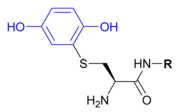
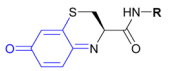
Fig. 2 Mass chromatogram of (A) Rf_8CYS (**1**) (*m*/*z* 567) and its adduct **3** (*m*/*z* 655) and (B) non-fluorous trapping reagent **2** (*m*/*z* 261) and its adduct **4** (*m*/*z* 349).

To determine the feasibility of the separation of the fluorous-tagged adduct from the biological matrix of the liver microsome, we used two methods: (i) fluorous solid-phase extraction (F-SPE) and (ii) direct injection.

In the F-SPE method (Fig. 3 and S9†), the deproteinized sample was loaded onto a fluorous silica gel, which carries only fluorous compounds. An 80% MeOH solution, which served as a fluorophobic solvent, was used to eliminate non-fluorous compounds. Subsequently, a fluorophilic solvent (*e.g.*, 100% methanol) was used to elute all the fluorous compounds. The mass chromatogram of the human liver microsome samples showed a significant decrease in the background after F-SPE compared with that before F-SPE. In contrast, the fluorous-tagged adduct **3** was detected by LC/MS, even after F-SPE. This suggests that the biological matrices in the human liver microsomes were effectively removed by F-SPE, and the fluorous-tagged adduct **3** was retained by the fluorous silica gel column.

In the direct injection method, the deproteinized samples were injected directly into an LC/MS equipped with a fluorous column for analysis (Fig. 4 and S10†). In the LC/MS chromatogram of the human liver microsome samples, most microsome matrix peaks were detected at an early retention time (<10 min). The fluorous adduct **3** was more strongly adsorbed on the fluorous column than the microsome matrices. Consequently, the

Table 1 Reaction of the trapping reagent Rf_8CYS (R = $-C_2H_4C_8F_{17}$) with hard and soft electrophiles in aqueous solution (PBS : methanol = 100 : 1)

Electrophile	(Type)	Adduct
	(Hard)	**5** 
	(Hard)	**6** 
	(Hard)	**7** 
	(Soft)	**8** 
	(Soft)	**9** 
		10 

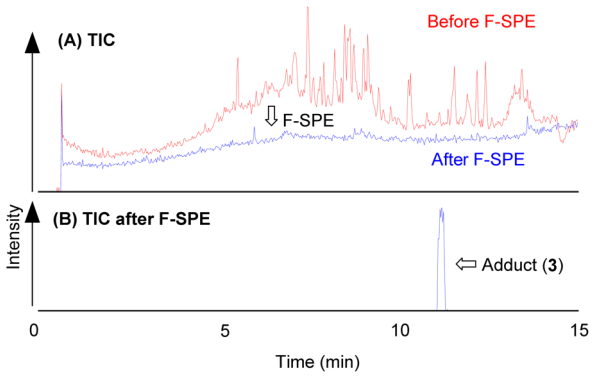


Fig. 3 Purification by fluorous solid-phase extraction (F-SPE). (A) Total ion chromatogram (*m/z* 100–1500) of the control sample [liver microsomes (1.0 mg mL^{-1}) fortified with a $NADPH^+$-generating system at 37 °C for 60 min] before and after purification using F-SPE. (B) MS chromatogram (*m/z* 655) of the fluorous-tagged adduct **3** after F-SPE.

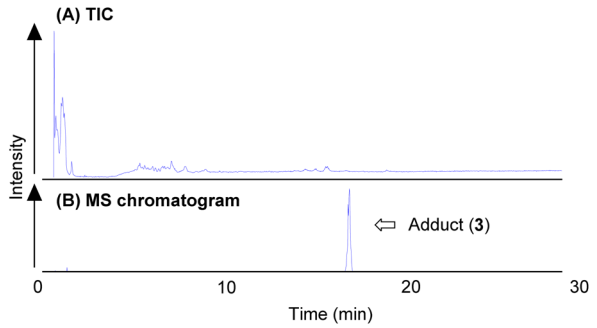


Fig. 4 (A) Total ion chromatogram of the control sample [liver microsomes (1.0 mg mL^{-1}) fortified with a $NADPH^+$-generating system at 37 °C for 60 min] using a fluorous LC column. (B) MS chromatogram (*m/z* 655) of the fluorous-tagged adduct **3** obtained using the direct injection method.

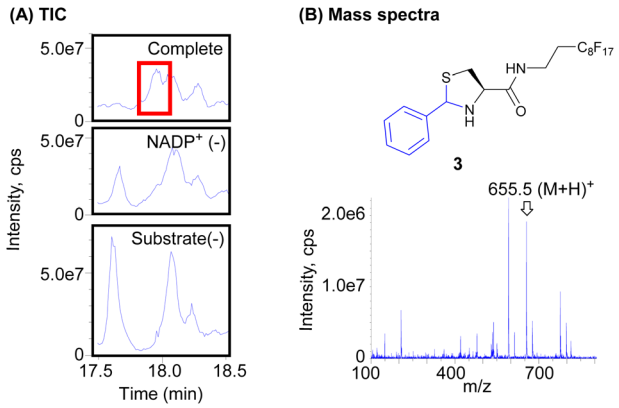


Fig. 5 Chromatograms and mass spectra of the microsomal incubation samples with benzyl alcohol and Rf_8CYS obtained using the direct injection method. Benzyl alcohol (150 μM) was incubated with Rf_8CYS (150 μM) in human liver microsomes (1.0 mg mL^{-1}) fortified with a NADPH + -generating system at 37 °C for 60 min. The samples with all the reagents were named complete samples, and the samples without a $NADPH^+$-generating system or benzyl alcohol were named control samples. (A) Total ion chromatogram (*m/z* 600–700): the red box in the complete sample indicates the adduct **3** formed from the benzaldehyde derived from benzyl alcohol. (B) Mass spectrum of the adduct **3** and its structure.

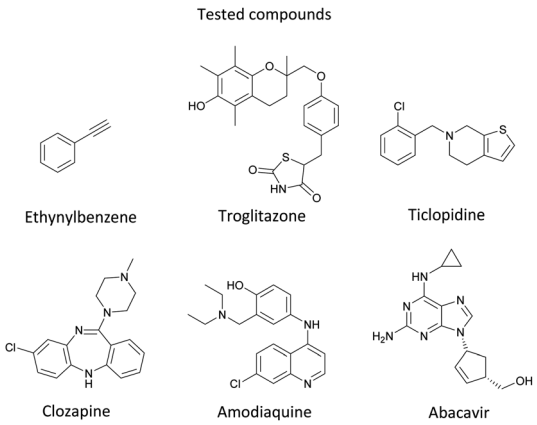
retention time of adduct **3** was longer. These results suggest that the fluorous-tagged adducts were readily separated from the biological matrices using both F-SPE and direct injection.

Next, metabolically produced benzaldehyde was detected using the trapping reagent Rf_8CYS (Fig. 5 and S11–S14†). Benzyl alcohol was incubated with human liver microsomes for 60 min in the presence of Rf_8CYS with and without the $NaDPH^+$-generating system. After the reaction was terminated, the reaction mixture was analyzed using the direct injection method. The expected benzaldehyde adduct **3** was successfully obtained ($[M + H]^+ = 655$). In contrast, **3** was not detected in the absence of $NADPH^+$, indicating that adduct **3** was derived from the metabolically produced benzaldehyde.

Finally, the RMs derived from ethynylbenzene and representative hepatotoxic drugs (troglitazone, ticlopidine, clozapine, amodiaquine, and abacavir) were detected using Rf_8CYS (Table 2 and Fig. S15–S21†). These compounds generate electrophilic RMs in human liver microsomes.

The drugs were incubated with Rf_8CYS, and the resulting mixtures containing RM adducts were analyzed using the direct injection method. Each compound was identified by their high-resolution MS (HRMS) *m/z* values and known adducts of cysteine or GSH (M + Rf_8CYS + O for ethynylbenzene; M + Rf_8CYS–2H for troglitazone, ticlopidine, clozapine, and amodiaquine; and M + Rf_8CYS–4H–O for abacavir).[12,13,17,18,23,25,40–42] For ticlopidine, an unprecedented metabolite adduct (M +

Table 2 High-resolution mass spectrometry (HRMS) and postulated composition of adducts produced from the microsomal incubation of compounds and Rf_8CYS[a]



Compound	Detected HRMS (m/z)	Composition of postulated adducts (calcd. m/z)
Ethynylbenzene	685.0825	M + Rf_8CYS + O (685.0812)
Troglitazone	1006.1870	M + Rf_8CYS–2H (1006.1852)
Ticlopidine	828.0793	M + Rf_8CYS–2H (828.0778)
	8.14.1171	M + Rf_8CYS–S + O (814.1163)
Clozapine	891.1544	M + Rf_8CYS–2H (891.1541)
Amodiaquine	920.1694	M + Rf_8CYS–2H (920.1694)
Abacavir	833.1683	M + Rf_8CYS–4H–O (833.1679)

[a] Each compound (150 μM) was incubated with Rf_8CYS (150 μM) in human liver microsomes (1.0 mg mL^{-1}) fortified with a $NADPH^+$-generating system at 37 °C for 60 min.

Rf_8CYS–S + O) was observed, possibly due to the improved MS sensitivity of Rf_8CYS.

Ticlopidine has a thiophene ring which is expected to metabolize into an α,β-unsaturated carbonyl compound based on a previous report.[43] Notably, the MS/HRMS data of unprecedent adduct M + Rf_8CYS–S + O (Scheme S3†) was consistent with the structure of proposed α,β-unsaturated carbonyl metabolite adduct **S5** (Fig. S22†). The HPLC retention time of the ticlopidine RM adduct on the C18 column (Fig. S21,† 7.79 min) was much shorter than that of **3** (Fig. S4,† 10.76 min). This confirms that the ticlopidine metabolite, which contains amine and hydroxyl groups, is a polar compound. Highly polar compounds tend to have low MS sensitivity in LC-electrospray ionization-MS (LC-ESI-MS) because their liquid-phase ions tend to be distributed inside the droplets. The low abundance of the ticlopidine metabolite and its poor MS sensitivity have previously prevented its detection. Nevertheless, the ticlopidine metabolite has been successfully detected *via* its adduct formed with a reagent bearing a highly hydrophobic fluorous tag.[32]

For further confirmation, we purified the adduct **S5** using the F-SPE method (Fig. S20†).

Metabolite detection using Rf_8CYS has three distinct advantages over the existing fluorous derivatization approach:[12] [a] operational advantage: the derivatization approach requires additional reactions and the associated reaction termination and centrifugation processes, leading to the loss of time and sample; [b] prevention of metabolite conversion: during the derivatization process, highly reactive metabolites undergo further conversion, owing to which the resulting compound may be mistaken for the corresponding metabolite; [c] applicability: the cysteine-type trapping reagent Rf_8CYS reacts with a wider range of functional groups than the previously reported thiol-type reagents.

Conclusions

We developed a novel fluorous cysteine-type trapping reagent, Rf_8CYS, that forms fluorous adducts of both soft and hard RMs in aqueous solvents. Fluorous-tagged adducts were separated from human liver microsome matrices using F-SPE and/or a fluorous HPLC column. Sensitive MS detection was achieved without the necessity for radiation or fluorescence detection. This novel method would be useful in the pharmaceutical sector for the structural determination of diverse RMs during drug discovery and development.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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