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Arylalkylidene Rhodanine with Bulky and Hydrophobic Functional Group as Selective HCV NS3 Protease Inhibitor

Wan Theng Sing,[a] Cheng Leng Lee,[a] Su Ling Yeo,[b] Siew Pheng Lim[b] and Mui Mui Sim[a,*]

[a]*Medicinal and Combinatorial Chemistry Laboratory, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Singapore*

[b]*Collaborative Antiviral Research Laboratory, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Singapore*

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Abstract—Arylalkylidene rhodanines **2(a–d)** inhibit HCV NS3 protease at moderate concentrations. They are better inhibitors of other serine proteases such as chymotrypsin and plasmin. However, the selectivity of arylmethylidene rhodanines (**8a**, **9a**) with bulkier and more hydrophobic functional groups increases by 13- and 25-fold towards HCV NS3 protease respectively.

Hepatitis C virus (HCV), identified in 1975, is classified as a new genus of the *Flaviviridae* family.[1] HCV infection leads to acute and chronic hepatitis, liver cirrhosis, and in some cases, hepatocellular carcinoma. Treatment with interferon is only effective in 20% of patients. The high rate of relapse, significant side effects, and exorbitant cost associated with the current treatments have, therefore, prompted extensive research in finding other effective means of therapy.

Several potential therapeutic targets have emerged following the sequencing of the HCV genome. The nonstructural protein 3 (NS3), a serine protease with a chymotrypsin-like fold,[2] plays an important role in cleaving the nonstructural HCV proteins that are necessary for HCV replication. Several attempts to seek HCV NS3 protease inhibitors have been reported. However, most of the nonpeptidic small molecule inhibitors reported are nonspecific and inhibit NS3 protease in the micromolar range.[3]

Rhodanine derivatives are known to possess biological activities such as anticonvulsant, antibacterial, antiviral and antidiabetic.[4] Sudo et al. have also reported rhodanine derivatives as HCV protease inhibitor.[3a] It is therefore an interesting and convenient core for further development of a selective NS3 protease inhibitor. Here, we report the synthesis of an arylalkylidene rhodanine library and the modification of the rhodanine side chain in an attempt to increase the selectivity towards the NS3 protease.

Synthesis

Rhodanine **1** was synthesized by reacting methyl thioglycolate with isothiocyanate at room temperature (Fig. 1). Knoevenagel condensation with aldehyde proceeded very smoothly to completion just by refluxing in ethanol for 6 h without using acid or base as condensing agent. The condensation of ketone with rhodanine **1** was achieved using ammonium acetate. Arylalkylidene rhodanine **2** was obtained as crystals precipitating out of the reaction mixture.[5] In some cases, black and sticky syrup obtained was washed with a small amount of CH_2Cl_2 and dried.

Of approximately 2000 compounds that were synthesized and characterized by ^{1}H NMR and MS, eight were randomly selected and their purities determined by HPLC. The results are summarized in Table 1. In general, aldehydes gave better condensation yields and purities as compared to ketones, and the more hindered ketones gave poorer yields than the less hindered ketones. The

*Corresponding author. Tel.: +65-874-7823; fax: +65-779-1117; e-mail: mcbsimmm@imcb.nus.edu.sg

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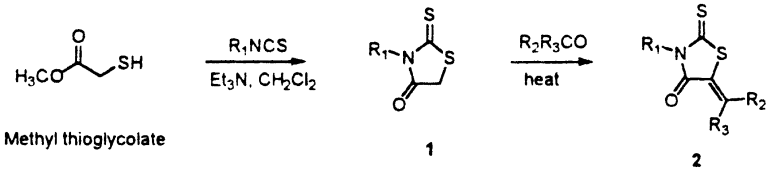
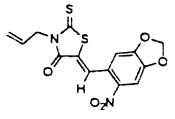
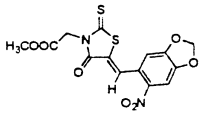
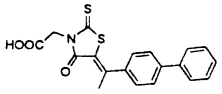
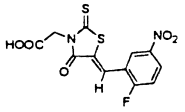
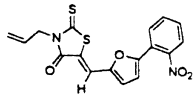
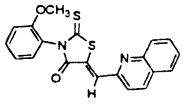
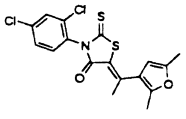
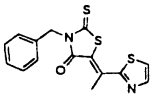


Figure 1.

impurities are mainly the unreacted aldehydes or rhodanines that co-precipitated out with the desired products. The thermodynamically stable *Z*-isomer was obtained for 5-benzylidene rhodanine.[4] Either exclusively *Z*-isomer or a mixture of the two geometrical stereoisomers with the *Z*-isomer being the predominant one (>75%) was obtained for the arylmethylidene rhodanines.

Crystal structure of NS3 protease complexed with a truncated NS4A cofactor peptide reveals that the S1 selectivity pocket is shallow and hydrophobic, and is primarily formed by the side chains of Phe154, Ala157 and Leu135.[7] Substrate specificity studies also demonstrate the importance of the acidic P6 residue to substrate recognition.[8] Based on the findings, it might be expected that a large inhibitor would be required to fully occupy the substrate-binding site. Charged and hydrophobic functional groups were therefore incorporated into arylalkylidene rhodanine **2** (Fig. 2).

Table 1. The yield[6a] and HPLC purity[6b] of compound **2**

Compounds		Yield	Purity
2a		72	88
2b		92	88
2c		67	91
2d		89	84
2e		89	80
2f		61	100
2g		62	73
2h		75	82

The *tert*-butyl *N*-(2-aminoethyl)-carbamate **3a** was dialkylated with methyl 4-(bromomethyl)-benzoate to give **4a**. The Boc protecting group was removed by TFA, and the free amine was mono-alkylated with 4-chlorobenzyl chloride to give **5a**. The amines **4b** and **4c** were synthesized by mono-alkylating **3a** and **3b** with 4-chlorobenzyl chloride and methyl 4-(bromomethyl)-benzoate, respectively. Amines **5a**, **4b** and **4c** were separately coupled to Fmoc-L-leucine using the standard peptide coupling condition. The Fmoc protecting group was then removed to yield **6**(**a**–**c**). Each step generally gave more than 60% yield. Finally, the amine **6** was activated by triphosgene and then coupled to **7** to give **8**.[9] The low isolated yields of **8a** and **8b** were primarily due to the formation of urea dimer (58% in the case of **8a**).

Arylalkylidene rhodanines **9**(**a**–**b**) with a sulfonamide functional group were also synthesized (Fig. 3). The less reactive 3-aminoacetophenone was first added dropwise to an ice-cooled dilute solution of biphenyl-4,4′-disulfonyl chloride in CH_2Cl_2, followed by another amine. The purified ketone was condensed as described above with rhodanine **1** to give **9**.

Results and Discussion

Arylalkylidene rhodanines **2** were tested in the NS3 protease assay in groups of 10 at 71 mM each (except **8** and **9** which were tested individually).[10–12] Pooled samples showing an inhibition of greater than 60% were screened individually at 71 μM again. The 50% inhibitory concentration (IC_{50}) of the active compounds was determined. To check for their inhibitory selectivity, the putative inhibitors were examined against two other serine proteases such as chymotrypsin and plasmin.[13] The results are summarized in Table 2.

Most of the arylalkylidene rhodanines **2** tested are not active except **2**(**a**–**d**), which are moderate and non-selective protease inhibitors. Like most reported non-peptidic small molecule inhibitors of NS3, all but **2c** inhibit chymotrypsin and plasmin better than NS3 protease.[3] Compounds **8a** and **9a** inhibit both plasmin and NS3 protease equally well, but they are not active against chymotrypsin. Compounds **2**(**a**–**d**), **8a** and **9a**

are also not active against elastase (data not shown). The IC_{50}'s of **8a** and **9a** against chymotrypsin are 13- and 25-fold (respectively) higher than that against NS3 protease. The fact that the IC_{50} of **8a** and **9a** against chymotrypsin and plasmin is about 10-fold higher than that of **2** suggests that selectivity for NS3 protease improves with a long hydrophobic side chain. This may also account for the activity but not the selectivity of **2b** against these serine proteases. Furthermore, the long hydrophobic side chain is likely to be important for the activity of **8a**, **9a** and **2c** towards NS3 protease as compared to **8(b–e)**, **9b** and **2(a, b, d)**.

In this paper, we have demonstrated that a long hydrophobic side chain is important for the selectivity as well as the activity of arylalkylidene rhodanine towards NS3

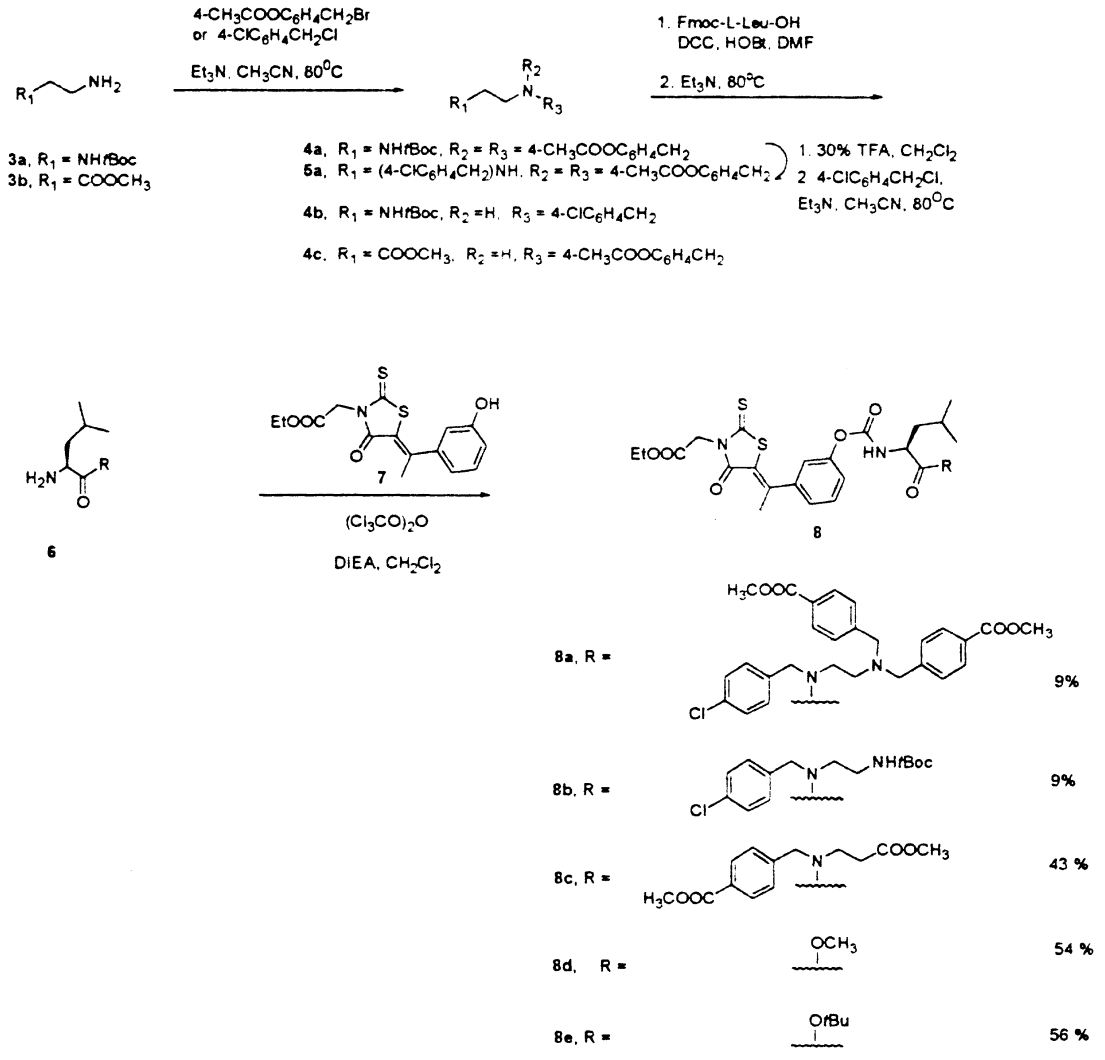


Figure 2.

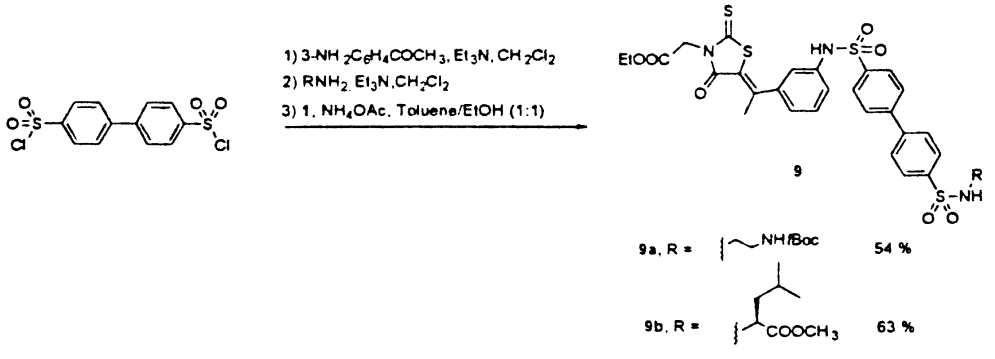


Figure 3.

Table 2. IC_{50} (μM)[a] of arylalkylidene rhodanines against serine proteases

Compounds	HCV NS3	Chymotrypsin	Plasmin
8a	16	210	20
9a	15	375	20
2a	40	27	< 1
2b	71	38	< 1
2c	20	23	7
2d	64	>200	13

[a]Values are means of two experiments.

protease. Our results provide more information required for further development of selective anti-HCV agents.

Acknowledgements

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5. Preparation of arylalkylidene rhodanine **2**: A mixture of isothiocyanate (0.11 mmol), methyl thioglycolate (0.1 mmol) and Et_3N (0.03 mmol) in CH_2Cl_2 was stirred for 1 h. Excess isothiocyanate was removed by amino-methylated polystyrene resin (0.015 mmol, Novabiochem). The solution was filtered and concentrated to give **1**. Rhodanine **1** was heated with an aldehyde (0.1 mmol) in anhydrous EtOH (200 μL) for 6 h at 80 °C. Alternatively, rhodanine **1** (0.1 mmol), ketone (0.1 mmol) and NH_4OAc (0.2 mmol) were refluxed in toluene (500 μL) for 3 days. The crystalline compound **2** was filtered, washed (EtOH and hexane, 100 μL each) and then dried. The average overall yield and HPLC purity are more than 70%.
6. (a) Yield was calculated based on the ratio of the mass of product to methyl thioglycolate expressed as a percentage. (b) HPLC analysis was performed at 254 nm with a Hypersil ODS C18 reverse-phase column (2.1×200 mm). A gradient (30% MeOH in H_2O to 100% MeOH in 10 min, followed by 100% MeOH for another 10 min) with flow rate 0.3 mL/min was employed.
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9. Preparation of arylalkylidene rhodanine **8**: Triphosgene (1/3 mol equiv) was added to a solution of **6(a–e)** and diisopropylethylamine (2 mol equiv) in anhydrous CH_2Cl_2 and stirred for 1 h followed by addition of **7** (1 mol equiv, prepared by reacting ethyl isothiocyanatoacetate with methyl 3-hydroxy-α-mercapto-β-methylcinnamate). The reaction mixture was stirred for another 1 h, concentrated and chromatographed on silica gel [hexane:ethyl acetate (75:25)] to yield **8(a–e)**. The two geometrical stereoisomers for **8a** ($E/Z = 1/4$) and **9a** ($E/Z = 1/5$) were inseparable by silica gel chromatography. HPLC purity for both **8a** and **9a** are 100%.
10. Cloning and preparation of HCV NS3: The full length HCV NS3 clone was obtained by PCR from the serum of a patient infected with genotype 1b of HCV. The NS3 protease fragment encoding only the protease domain (1-181aa) was then obtained by PCR from the above clone. The fragment was then cloned into the pET15B vector. The HIS tagged fusion protein was transformed and expressed in BL21 (DE3) bacterial cells. Expression and purification was based on a modified method.[11] The enzyme was quantitated by densitometry with bovine serum albumin as standards. Fusion protein was frozen in 10 μL aliquots at −70 °C.
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12. HCV NS3 protease assay: The peptide 4A (NH_2-LTTGSVVIVGRIILSGRPAVVPD-COOH) from amino acids 18–40 of NS4A at 20 μM final concentration was used to enhance NS3 protease activity. pETNS3 (0.01 μg) was preincubated with pep4A and chemical compounds diluted in assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 10 mM DTT and 15% glycerol) at room temperature for 15–30 min. The substrate 5A5B was generated by cloning 5A5B cleavage site (EEASEDVVPCSMSYTWTGACCFGTM) into the pGEX4T-1 vector. The substrate was expressed and purified in bacterial cells. GST beads containing 3 μg of substrate 5A5B were then added to start the reaction. Assay was performed at 37 °C for 15 min or at times giving less than 40% conversion. *N*-Tosyl-L-phenylalanine chloromethyl ketone at 400 μM was used as the positive control. SDS loading buffer was added to stop the reaction. Ten microliters of sample were loaded and run in 15% polyacrylamide gels. Bands were visualized with Coomassie Blue and the intensity of the bands was quantitated using the densitometer.
13. Chymotrypsin and plasmin assay: The fluorogenic assays were performed according to the manufacturer's directions from Molecular Probes (Enchek Protease Molecular probes) that use casein as the substrate. 0.005U of α-chymotrypsin and 0.002U plasmin were used per assay. All reactions were performed at 30 °C in black 96-well plates (Nunclon) and stopped at the linear portion of the enzyme reactions. The enzymes α-chymotrypsin was purchased from Sigma (Cat No.: 7762) while plasmin was purchased from Calbiochem (Cat No.: 527621).