Synthesis and *in vitro* activity towards *Mycobacterium tuberculosis* of L-serinyl ester and amino derivatives of pyrazinoic acid

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Reactions between either L-serine methyl ester hydrochloride (**1**), or the cbz derivative, methyl (*S*)-(+)-2-(benzyloxycarbonylamino)-3-hydroxypropanoate (**2**), and pyrazinoyl chloride (**3**), have been studied. Methyl (*S*)-(+)-2-benzyloxycarbonylamino-3-[(pyrazinecarbonyl)oxy]propionate (**4**), methyl (*S*)-(+)-3-hydroxy-2-[(pyrazine-2-carbonyl) amino]propionoate (**7**), methyl 2-[(pyrazinecarbonyl)amino]acrylate (**8**) were obtained. Additional products, methyl (*S*)-(+)-2-benzyloxycarbonylamino-3-formyloxypropionoate (**5**) and methyl (*R*)-(+)-2-benzyloxycarbonylamino-3-chloropropionoate (**6**), were isolated from reaction of **2** with **3**, in the presence of DMF remaining from the preparation of **3**, from pyrazinecarboxylic acid. The coupling of pyrazinecarboxylic acid with **1**, in the presence of DCC was prevented by the formation of the unreactive adduct between DCC and pyrazinoic acid. The compounds were tested against *M. tuberculosis:* compounds (**8**) and (**6**) exhibited a MIC (μg/ml) value of 50 and 100, respectively, compared to the MIC value of 100 for the first line TB drug, pyrazinamide. The confirmation of the structure of (**8**) was obtained via X-ray crystallography.

Keywords: synthesis, tuberculosis, L-serine derivatives, pyrazinoic acid derivatives, crystal structure, didehydroamino acid

Tuberculosis (TB) has once again become a major worldwide public health problem and was declared a global health emergency in 1993 by the World Health Organisation (WHO).[1]

Together with rifampicin and isoniazid, pyrazinamide (Fig. 1) is a currently used first-line drug. Its inclusion in TB treatment regimen decreases the duration of the treatment from twelve to six months. Pyrazinamide is a pro-drug, only active in acidic media on hydrolysis to pyrazinoic acid, the active metabolite, produced by the mycobacterial enzyme pyrazinamidase. The inhibition of the growth of *Mycobacterium tuberculosis* by pyrazinoic acid is based on blocking the synthesis of fatty acids by deactivating the fatty acid synthetase I enzyme.[2]

Among second-line TB drugs are the L-serine derivatives, cycloserine and terizidone, shown in Fig. 2.

As part of our research for new compounds with anti-TB activities, we initiated a study of compounds containing moieties of both first-line and second-line TB drugs, namely pyrazinoic acid and serine derivatives. We now wish to report our findings, including *in vitro* test results.

Results and discussion

Routes to serinyl-pyrazine derivatives were attempted using either L-serine methyl ester hydrochloride (**1**), or the cbz derivative,[3] methyl (*S*)-(+)-2-(benzyloxycarbonylamino)-3-hydroxypropanoate (**2**) formed from L-serine as shown in

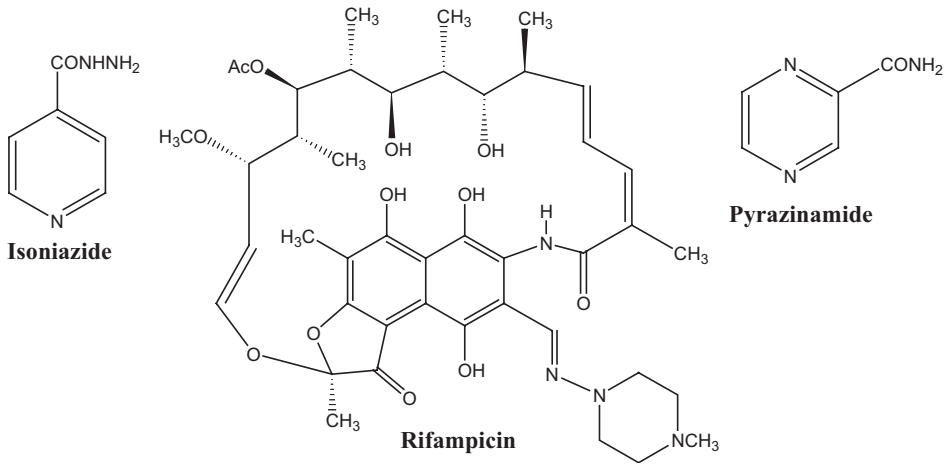

Fig. 1

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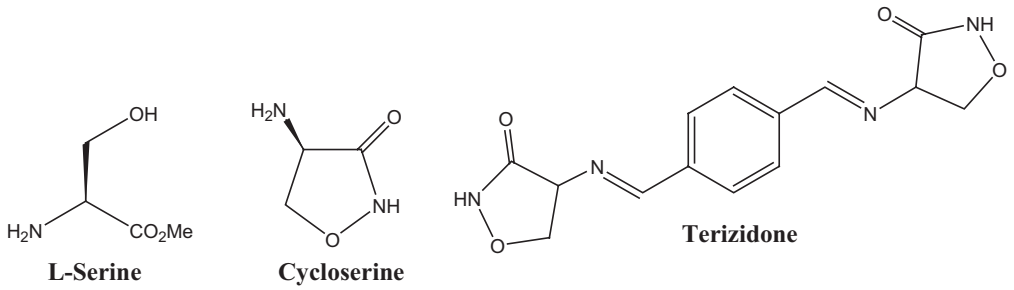


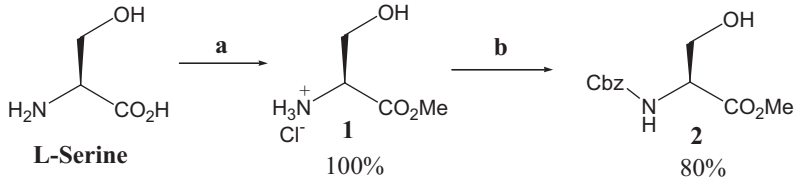
Fig. 2

Scheme 1, and pyrazinoyl chloride or methyl pyrazinoate.

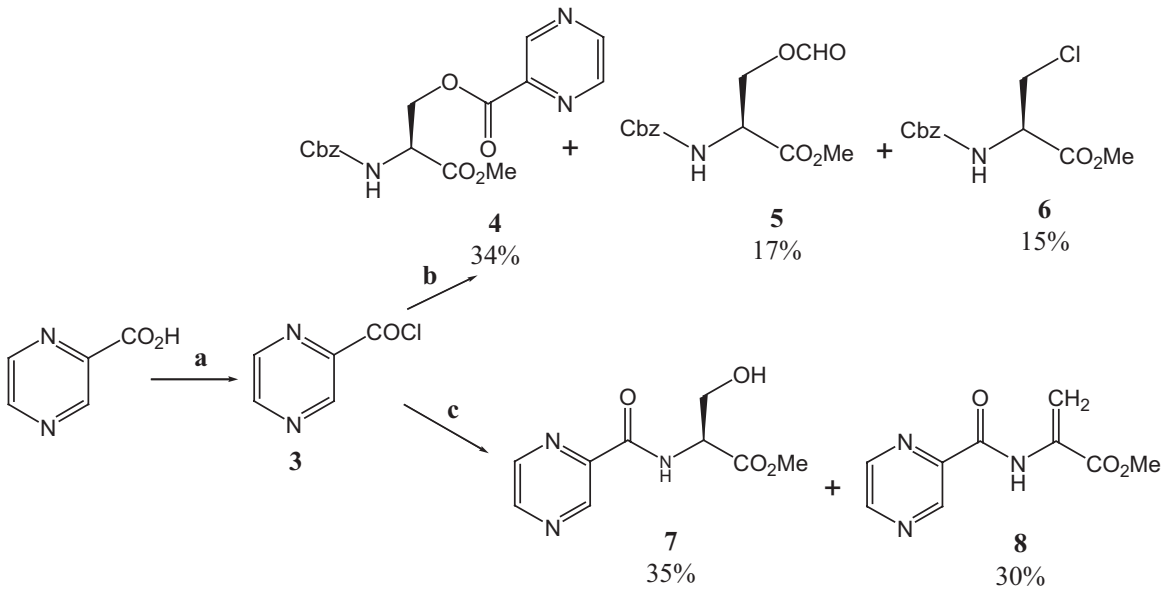
Pyrazinoyl chloride (**3**), was generated from pyrazinecarboxylic acid on treatment with excess $SOCl_2$ in dichloromethane, in the presence of a catalytic amount of DMF. However, attempts to isolate pyrazinoyl chloride completely from the reaction mixture on removal of all volatiles only led to decomposition and tar formation. Hence, evaporation was only used to concentrate the reaction mixture to 1–2 ml in volume, which consequently left some DMF and maybe also some $SOCl_2$ or HCl, admixed with the acid chloride.

The major product, albeit in a low 34% yield, from reaction of such a concentrated impure solution of pyrazinoyl chloride (**3**), with **2** was the ester, methyl (*S*)-(+)-2-benzyloxycarbonylamino-3-[(pyrazinecarbonyl)oxy]propionate (**4**),[4] see Scheme 2, *via* reaction at the free hydroxyl group of **2**. Other products isolated from the reaction mixture by column chromato-graphy were methyl (*S*)-(+)-2-(benzyloxy-carbonylamino)-3-formyloxy-propionate (**5**), in 17% yield, and methyl (*R*)-(+)-2-benzyloxycarbonylamino-3-chloro-propionoate (**6**), in 15% yield. Characterisations of the products were generally achieved using NMR and IR spectroscopies. The three distinct carbonyl groups in **4** exhibit three C=O peaks in the IR spectrum at 1760 (CO_2Me), 1725 (CO_2R) and 1690 (CONH) $cm^{-1}$and three C=O absorptions at 169.8 (CO_2Me), 163.0 (CO_2R) and 156.1 (CONH) ppm in the ^{13}C NMR spectrum. Product **5** is formed from reaction of the hydroxyl in **2** with the residual DMF left amongst the pyrazinoyl chloride, while compound **6** is formally obtained by chloride substitution of the OH group in **2**.

The reaction of another sample of pyrazinecarbonyl chloride, obtained in a similar manner as indicated above, with L-serine methyl ester hydrochloride (**1**), produced methyl (*S*)-(+)-3-hydroxy-2-[(pyrazine-2-carbonyl)amino] propionoate (**7**), methyl 2-[(pyrazinecarbonyl)amino]acrylate (**8**), in yields of 35 and 30%, respectively, (Scheme 2). The two distinct carbonyl groups in **7** exhibited two C=O peaks in the IR spectrum at 1743 (CO_2CH_3) and 1674 (CONH) cm^{-1} and two C=O absorptions at 171.2 (CO_2Me) and 164.0



Scheme 1 (a) $SOCl_2$, MeOH, RT, 24 h; (b) $NaHCO_3$, H_2O, $PhCH_2OCOCl$, 0°C, 3 h.



Scheme 2 (a) $SOCl_2$, DMF, DCM, RT, 3 h; (b) DMF, Et_3N, THF, **2**, RT, 5 h; (c) DMF, Et_3N, THF, **1**, RT, 5 h.

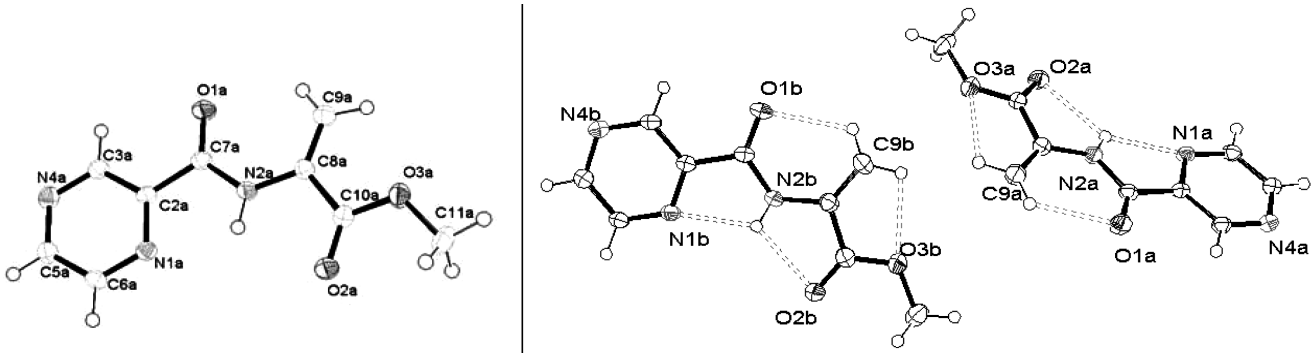


Fig. 3 (a). Atom arrangements and numbering scheme for one, molecule A, of the two independent molecules of **8**. (b) Intramolecular H-bonding in **8**, for both the independent molecules. Probability elipsoids drawn at the 50% level.

(CONH) ppm in the ^{13}C NMR spectrum. The OH group is apparent from the stretching frequency at 3384 cm^{-1} in the IR spectrum and the absorption at 2.37 ppm exchangeable with D_2O in the ^1H NMR spectrum. As in **6**, the NH proton in **7** appears as a doublet at 8.62 ppm in the ^1H NMR spectrum. The formation of **7** indicates the much greater reactivity of the amino group relative to the hydroxyl group in **1** towards the acyl chloride.

The two distinct carbonyl groups in **8** exhibited two peaks in the IR spectrum at 1744 (CO_2Me) and 1636 (CONH) $cm^{-1}$and two absorptions at 163.6 (CO_2Me) and 161.0 (CONH) ppm in the ^{13}C NMR spectrum. The ^1H NMR spectrum showed the NH proton as a singlet at 10.2 ppm, and the two protons of the double bond as broad singlet absorptions at 6.85 and 6.07 ppm. $J(H_A,H_B)$ couplings for H_AH_BC = R(R') are generally in the range of 2-4 Hz; clearly these were not resolved in our spectrum, nor in the reported ^1H NMR spectra of other H_2C =C(NHCOR)CO_2H compounds [see for example: ref. 5] The formation of the alkenyl compound **8**, in which the C =C double bond is in conjugation with the CO_2Me group, is derived from dehydration of the alcohol **7**, facilitated by the acidic reaction conditions. Phenyl and picolinyl analogues of **8**, PhCONHC(=CH_2)CO_2Me and 2-pyridinyl-CONHC (=CH_2)CO_2Me were indicated in a patent to be readily obtained from successive treatment of ArCONHCH(CH_2OH)CO_2Me (Ar = Ph or 2-pyridinyl) with $SOCl_2$ and a base.[6]

Compound **8** is an didehydroamino acid. Didehydroamino acids, in general have been synthesised by several routes,[7] including β-elimination of serine derivatives. They have been shown to be useful intermediates in amino acid and peptide synthesis and are constituents of a number of naturally occurring antibiotic and phytotoxic peptides.[7-11]

Confirmation of the structure of **8** was obtained *via* X-ray crystallography. Figure 3a shows the atom arrangements and the numbering scheme for one, molecule A, of the two independent molecules of **8**: the other molecule B, which has B in place of A in the numbering scheme, is very similar as shown by geometric parameters available from CCDC.

The core atoms of the side chains, C7, N2, C8 and C10 are near co-planar with their attached pyrazine rings, angles between the best planes for these side chain atoms and the ring atoms being 3.74(2) and 2.70(1)°, respectively in molecules A and B. Extensive strong intramolecular H-bonding, as shown in Fig. 3b and as indicated by the PLATON analysis, cement the particular conformation for each independent molecule. Intermolecular C–H---O H-bonding link the two independent molecules as shown in Fig. 4. Further weak intermolecular hydrogen bonding involving the methyl hydrogens, were also indicated by the PLATON program.

The lack of high yielding reactions with pyrazinoyl chloride, led to the attempted use of methyl pyrazinecarboxylate, formed in an one-pot synthesis from pyrazinecarboxylic acid, $SOCl_2$ and MeOH.[13] However, methyl pyrazinecarboxylate was found to be unreactive towards L-serine methyl ester hydrochloride, **1**, under a number of conditions, including refluxing THF in the presence of Et_3N, and under 50 bar pressure.

The coupling reaction of pyrazinecarboxylic acid with L-serine methyl ester hydrochloride in the presence of dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt) was also attempted, the target being **7** in a higher yield than obtained from the acid chloride. DCC, which activates the acid on forming an adduct, has proved especially successful in aiding amide synthesis from aminoacid derivatives in peptide and nucleotide chemistry. However, alternative products have been frequently reported in attempted DCC assisted coupling reactions between other carboxylic acids, RCO_2H, and an amine or an alcohol. These alternative products for amine reactions include anhydrides [RCO_2OCR], ureas [cyclohexyl-NHCONH-cyclohexyl] and acylureas [cyclohexyl-NHCON (COR)NH-cyclohexyl].[14-15] From the DCC, HOBt, pyrazine-carboxylic acid and L-serine methyl ester hydrochloride reaction mixture in CH_2Cl_2, the major product isolated in ~70% yield was the acylurea derivative, **9**, obtained by rearrangement of the initial pyrazinecarboxylic acid/DCC adduct (**10**), and a little cyclohexyl-NHCONH-cyclohexyl: no amide **7** was detected. The acylurea derivatives, such as **9**, unlike the initial adducts, such as **10**, are unreactive towards amines and alcohols.

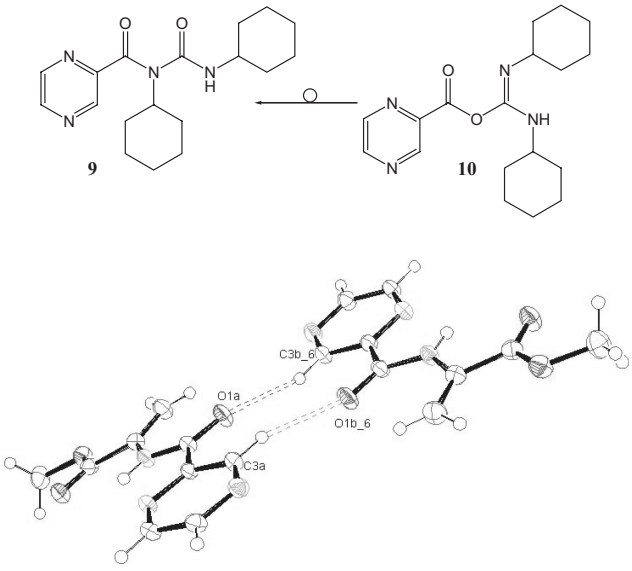


Fig. 4 Intermolecular C–H---O interactions linking the two independent molecules of **8**.

Interestingly, Ljungvist and Folkers reported[16] the successful formation of the *p*-nitrophenyl pyridine-2-carboxylate in 70% yield from *p*-nitrophenol and pyridine-2-carboxylic acid in the presence of DCC in CH_2Cl_2, but only 16% was obtained in DMF, along with 30% of 1,4-dicyclohexyl-2-(2-pyridylcarbonyl) semicarbazide. In contrast, good yields of the *p*-nitrophenyl esters of 3- and 4-pyridinecarboxylic acids were obtained even in DMF. Such results for similar nitrogen heteroaromatic acids show how small changes effect the outcome of these DCC assisted reactions.

Biological activity

The anti-mycobacterial activities of the compounds were assessed against *M. tuberculosis* ATTC 27294 using the micro plate Alamar Blue assay (MABA).[17] The results are listed in Table 1. Compounds **8** and **6** exhibited a MIC (μg/ml) value of 50 and 100, respectively, which can be compared with the MIC value of 100 for the first line TB drug, pyrazinamide.

Experimental

General

Melting points were determined on a Buchi apparatus and are uncorrected. IR spectra were determined using a Thermo Nicolet Nexus 670 spectrometer in potassium bromide discs. Mass spectra recorded on a Q-TOF mass spectrometer Typical ESI-MS (Micromass, Manchester, UK); NMR spectra were obtained at room temperature with a Bruker Avance 500 spectrometer operating at 500.00 MHz (^{1}H) and 125.0 MHz (^{13}C), in DMSO-d_6 solutions. Optical rotation was measured with a Polartronic NH8 polarimeter at ~25°C. Thin layer chromatography was carried out on silica gel plates, using ethyl acetate/methanol mixtures as eluents, with products detected by UV light. For column chromatographic purification, column grade silica gel 60 (0.063–0.200 mm mesh size) was employed.

Synthesis

L-Serine methyl ester hydrochloride salt (**1**): To a stirred solution of thionyl chloride (69.5 ml, 0.95 mol) in methanol (400 ml) at 0°C was added L-serine (20 g, 0.19 mol). The reaction mixture was stirred for 24 h at room temperature, the solvent removed to give L-serine methyl ester hydrochloride salt, **1** (147.2 g) in quantitative yield. m.p. 163.1°C (Lit.: 163°C[26]). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.60 (s, 3H, H_3N^+), 5.64 (s, 1H, OH), 4.08 (t, *J* = 4 Hz, 1H, CH), 3.82 (d, *J* = 4 Hz, 2H, CH_2), 3.73 (s, 3H, CH_3).^{13}C NMR (100 MHz, DMSO-d_6) δ: 168.5, 59.5, 54.4, 52.8.IR (cm^{-1}; KBr): 3362 (O-H); 1743 (CO_2Me).

Methyl (S)-(+)-2-(benzyloxycarbonylamino)-3-hydroxypropanoate (**2**): To a reaction mixture containing L-serine methyl ester hydrochloride salt (**1**), (13.2 g, 0.11 mol), water (100 ml), diethyl ether (75 ml) and sodium bicarbonate (48 g, 0.55 mol) at 0°C was added dropwise benzyl chloroformate (21 ml, 0.15 mol). After 2 h at 0°C and 1 hour at room temperature, the reaction was quenched with pyridine, and water was added to solubilise all salts. The organic layer was washed with HCl (2.5N), dried ($MgSO_4$), filtered and concentrated. The residue was chromatographed (10%→30% EtOAc/ hexanes) affording **2** as a colourless oil (22.4 g, 80% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.32 (m, 5H, Ph), 5.71(s, 1H, NH), 5.13 (s, 2H, C$\underline{H}_2$Ph), 4.45 (m, 1H, CH), 4.00 (dd, *J* = 11.2 and 3.0, 1H, C$\underline{H}$'OH), 3.92 (dd, *J* = 11.2 and 3.0, 1H, C$\underline{H}$OH), 3.78 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.8, 157.0, 136.7, 129.2, 128.9, 128.7, 67.8, 63.7, 56.7, 53.3. IR (cm^{-1}, KBr): 3358 (O-H), 1778 (CO_2Me), 1653 (CONR). GC/MS: *m/z* $[M]^+$: 253. $[\alpha]_D^{30}$: + 10.0 (c = 1.0, $CHCl_3$)

Table 1 The *in vitro* activity of compounds against *M. tuberculosis* H_{37}Rv strain (ATCC 27294, susceptible both to rifampin and isoniazid)

Compound	MIC (μg/ml)
2	Resistant
4	Resistant
5	Resistant
6	100
7	Resistant
8	50
9	Resistant
Pyrazinamide	100

Reaction between pyrazinecarbonyl chloride (**3**)*, and methyl (S)-(+)-2-(benzyloxycarbonyl)-3-hydroxypropanoate* (**2**)*. Formation of methyl (S)(+)-2-benzyloxycarbonylamino-3-(pyrazinecarbonyloxy) propionate*(**4**),*methyl(S)-(+)-2-benzyloxycarbonylamino-3-formyloxy-propionoate* (**5**)*, and methyl (R)-(+)-2-benzyloxycarbonylamino-3-chloro-propionoate* (**6**).

Pyrazinecarbonyl chloride (**3**),was prepared from the reaction of pyrazinecarboxylic acid (1 g, 8.06 mmol), thionyl chloride (2.4 ml, 32.24 mmol) and DMF (0.06 ml, 0.81 mmol) in dichloromethane (30 ml) at room temperature, under stirring and nitrogen atmosphere. After 3–4 hours, the reaction mixture was concentrated by evaporation to 1–2 ml and the crude mixture was used as such in the next stage.

To the stirred solution of methyl (*S*)-(+)-2-(benzyloxycarbonyl)-3-hydroxypropanoate, 2, 7.25 mmol) and triethylamine (1.5 ml, 10.9 mmol) in anhydrous THF (25 ml) at 0°C was added pyrazinecarbonyl chloride, **3**, concentrate. The reaction mixture was stirred for 5 hours at RT, quenched with water (20 ml), and extracted with ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel, eluting with hexane: ethyl acetate gradient (up to 20%) to give methyl (*S*)-(+)-2-(benzyloxycarbonylamino)-3-(pyrazinecarbonyloxy) propionate (**4**), methyl (*S*)-(+)-2-(benzyloxycarbonylamino)-3-(formyloxy)-propionoate (**5**), and methyl (*R*)-(+)-2-(benzyloxy-carbonylamino)-3-chloropropionoate (**6**).

Methyl (S)-(+)-2-(benzyloxycarbonylamino)-3-(pyrazinecarbonyl-oxy)propionate (**4**): Yield: 34%, as a colourless oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.22 (d; *J* = 1.0; 1H; H_3); 8.90 (d; *J* = 2.0; 1H; H_6); 8.83 (dd; *J* = 2.0 and 1.0; 1H; H_5); 8.12 (d; *J* = 7.5; 1H; NH); 7.32 (m; 5H; Ph); 5.06 (s; 2H; C$\underline{H}_2$Ph); 4.66 (m; 2H; C$\underline{H}$'OCO and CH); 4.58 (dd; *J* = 12.5 and 4.5; 1H; C$\underline{H}$OCO); 3.69 (s; 3H; CH_3). ^{13}C NMR (100 MHz; DMSO-d_6) δ: 169.8; 163.0; 156.1; 148.2; 145.9; 144.9; 142.7; 136.7; 128.4; 127.9; 127.8; 65.8; 64.3; 52.9 52.4. IR (cm^{-1}; KBr): 1760 (C(O)OCH_3); 1725 (C(O)OR; 1690 (C(O)NR). $[\alpha]_D^{30}$: + 38.7 (c = 1.0, $CHCl_3$). HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{17}H_{17}N_3O_6{\cdot}Na^+$, 382.1015; found, 382.0982.

Methyl (S)-(+)-2-(benzyloxycarbonylamino)-3-(formyloxy)-propionoate (**5**): Yield: 17%, as a colourless oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.21 (s; 1H; OCOH); 7.97 (d; *J* = 8; 1H; NH); 7.34 (m; 5H; Ph); 5.06 (s; 2H; C$\underline{H}_2$Ph); 4.46 (m; 2H; C$\underline{H}$'OCOH and CH); 4.26 (dd; *J* = 11.2 and 6.4; 1H; C$\underline{H}$OCOH); 3.67(s; 3H; CH_3). ^{13}C NMR (100 MHz; DMSO-d_6) δ: 170.3; 162.2; 156.6; 137.0; 128.9; 128.4; 128.1; 66.3; 62.6; 53.3; 52.8. IR (cm^{-1}; KBr): 1750 (C(O)H); 1719 (C(O)OCH_3); 1692 (C(O)NR). $[\alpha]_D^{30}$: + 10.0 (c = 1.0, $CHCl_3$). HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{13}H_{15}NO_6{\cdot}Na^+$, 304.0797; found, 304.0790.

Methyl (R)-(+)-2-(benzyloxycarbonylamino)-3-chloropropionoate (**6**): Yield: 15%. m.p. 53.0°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.98 (d; *J* = 8; 1H; NH); 7.33 (m; 5H; Ph); 5.07 (s; 2H; CH_2Ph); 4.49 (m, 1H; CH); 3.90 (dd; *J* = 11.5 and 4.5; 1H; CH'Cl); 3.83 (dd; *J* = 11.5 and 7.5; 1H; CHCl); 3.36 (s; 3H; CH_3). ^{13}C NMR (100 MHz; DMSO-d_6) δ: 169.9; 156.4; 137.0; 128.7; 128.3; 128.0; 66.1; 55.6; 52.8; 44.1. IR (cm^{-1}; KBr): 1746 (C(O)OCH_3); 1686 (C(O)NR). $[\alpha]_D^{30}$: + 39.3 (c = 1.0, $CHCl_3$). HRMS-ESI: $[M + Na]^+$ calcd for $C_{12}H_{14}ClNO_4{\cdot}Na^+$, 294.0509; found, 294.0520.

Reaction between pyrazinecarbonyl chloride (**3**)*, and L-serine methyl ester hydrochloride salt* (**1**)*. Formation of methyl (S)-(+)-3-hydroxy-2-[(pyrazinecarbonyl)amino]propionoate* (**7**)*, and methyl 2-[(pyrazinecarbonyl)-amino]acrylate* (**8**)

Pyrazinecarbonyl chloride (**3**), was prepared from pyrazinecarboxylic acid (1 g, 8.06 mmol), thionyl chloride (2.4 ml, 32.24 mmol) and DMF (0.06 ml, 0.81 mmol) in dichloromethane (30 ml) at room temperature.

To the stirred solution of L-serine methyl ester hydrochloride salt (**1**)(860 mg, 7.25 mmol) and triethylamine (1.5 ml, 10.9 mmol) in anhydrous THF (25 ml) at 0°C was added pyrazinecarbonyl chloride (**3**) concentrate from above. The reaction mixture was stirred for 5 h at RT, quenched with water (20 ml), and extracted with ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel, eluting with hexane: ethyl acetate gradient (up to 20%) to give **7** and **8** in 35 and 30% yields, respectively.

Methyl (S)-(+)-3-hydroxy-2-[(pyrazinecarbonyl)amino]propionoate (**7**): Yield: 35%, as a colourless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.38 (s; 1H; H_3); 8.78 (d; *J* = 2; 1H; H_5 or H_6); 8.62 (d; *J* = 7.5;

1H; NH); 8.58 (d; J = 2; 1H; H_5 or H_6); 4.89 (m; 1H; CH); 4.15 (dd; J = 11.5 and 3.5; 1H; CH'OH); 4.06 (dd; J = 11.5 and 3.5; 1H; CHOH); 3.84 (s; 3H; CH_3); 2.37 (s; 1H; OH). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 171.2; 164.0; 148.2; 145.0; 144.6; 143.5; 63.8; 55.4; 53.6. IR (cm^{-1}; KBr): 3384 (O-H); 1743 (C(O)OCH_3); 1674 (C(O)NR). $[\alpha]_D^{30}$: + 33.0 (c = 1.0, $CHCl_3$). HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_9H_{11}N_3O_4$. Na^+, 248.0647; found, 248.0636.

Methyl 2-[(pyrazinecarbonyl)amino]acrylate (**8**): Yield: 30%. m.p. 95.5°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 10.2 (s; 1H; NH); 9.44 (s; 1H; H_3); 8.80(d; J = 2; 1H; H_5 or H_6); 8.61 (d; J = 2; 1H; H_5 or H_6); 6.85 (s; 1H; CCH'); 6.07 (s; 1H; CCH); 3.92 (s; 3H; CH_3). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 163.6; 161.0; 147.1; 143.9; 143.6; 142.2; 130.3; 109.4; 52.5. IR (cm^{-1}; KBr): 1744 C(O)OCH_3; 1636 (C(O)NR). HRMS-ESI (*m/z*): $[M + H]^+$ calcd for $C_9H_9N_3O_3$. H^+, 208.0722; found, 208.0728.

1,3-Dicyclohexyl-1-(pyrazinecarbonyl)urea (**9**): To a stirred solution of pyrazinecarboxylic acid (500 mg, 4.1 mmol) in anhydrous CH_2Cl_2 (25 ml) were added DCC (645 mg, 3.15 mmol) and HOBt, 1-hydroxybenzotriazole (13 mg, 0.1 mmol). After 2 h at room temperature, the precipitate of 1,3-dicyclohexylurea was removed by filtration, and the filtrate was poured into saturated aqueous $NaHCO_3$ solution (20 ml) and extracted with EtOAc (3 × 20 ml). The combined organic layers were dried ($MgSO_4$), filtered and concentrated. The residue was chromatographed (10%→50% EtOAc/hexanes) providing compound **9** (707 mg, 68%).

Yield: 68%. m.p. 164.5°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.99 (d; J = 1.6; 1H; H_3); 8.65 (d; J = 2.4; 1H; H_6); 8.51 (dd; J = 2.4 and 1.6; 1H; H_5); 5.94 (s; 1H; NH); 4.22 (m; 1H; NCH); 3.56 (m; 1H; NHCH); 1–2 (m; 20H; cyclohexyl). ^{13}C NMR (100 MHz; $CDCl_3$) δ: 166.7; 154.2; 149.6; 146.6; 145.4; 143.2; 57.2; 50.6; 33.1; 31.4; 26.8; 26.0; 25.9; 25.3. IR (cm^{-1}; KBr): 1698 (CONH) and 1677 (CON). HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{18}H_{26}N_4O_2$. Na^+, 353.1953; found, 353.1967.

Crystallography

The crystals of **8** were grown from EtOH. The intensity data were collected at 120K using synchrotron radiation on a Bruker SMART APEX2 CCD diffractometer at Daresbury SRS station 9.8. The process of data collection was accomplished by means of the program Bruker APEX2.[18] Cell refinement and data reduction were accomplished by the program BrukerSAINT.[19] The structures were solved by direct methods in SHELXS-97[20] within the OSCAIL suite of programs[21] and refined in SHELXL-97[22]. All H atoms were located in difference maps, and then treated as riding atoms. The H atoms bonded to C atoms were placed geometrically, with Uiso(H) = 1.5Ueq(C) for methyl groups and Uiso(H) = 1.2Ueq(C) for others. The H atoms bonded to N and O atoms were allowed to ride at the sites located from difference maps with iso(H) = 1.2Ueq(N) and Uiso(H) = 1.2Ueq(O) respectively. PLATON was used for the data analysis, including conformational and H-bonding analysis, was performed using PLATON.[12] The program ORTEP-3 for Windows was used to obtain the figures.[23] Crystal data and structure refinement details are listed in Table 2. "CCDC 633020 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif".

Table 2 Crystal data and structure refinement for **8**

Empirical formula	$C_9H_9N_3O_3$
Formula weight	207.19
Temperature, K	120(2)
Wavelength, Å	0.69050
Crystal system, space group	Monoclinic, C2/c
Unit cell dimensions	
a, Å	41.838(7)
b, Å	3.7273(6)
c, Å	24.707(4)
β, °	101.835(2)
Volume, Å^3	3771.0(11)
Z	16
Density (calculated), Mg/m^3	1.460
Absorption coefficient, mm^{-1}	0.113
F(000)	1728
Crystal size, mm	0.10 × 0.05 × 0.05
Theta range for data collection	2.90 to 29.55
Index ranges, °	–58< = *h* < = 58
	–5< = *k* < = 5
	–35< = *l* < = 28
Reflections collected	14573
Independent reflections	5391 [R(int) = 0.0567]
Reflections observed (>2 sigma)	3975
Data Completeness	0.934
Absorption correction	None
Refinement method	Full-matrix least-squares
on F^2	
Data/restraints/parameters	5391/0/292
Goodness-of-fit on F^2	1.029
Final R indices [I > 2 sigma(I)]	R1 = 0.0546 wR2 = 0.1452
R indices (all data)	R1 = 0.0745 wR2 = 0.1596
Largest diff. peak and hole, e. Å^3	0.309 and –0.265

Biological testing

The antimycobacterial activity to the compounds was assessed against *M. tuberculosis* ATTC 27294 using the micro plate Alamar Blue assay (MABA).[17] This methodology is nontoxic, temperature–stable reagent and show good correlation with proportional method and BACTEC radiometric method.[24-25] Sterile deionised water (200 µl) were added to all outer-perimeter wells of sterile 96 well plates (Falcon, 3072: Becton Dickinson, Lincoln Park, NJ, USA) to minimise evaporation of the medium in the test wells during incubation. The 96 plates received 100µl of the Middlebrook 7H9 broth (Difco laboratories, Detroit, MI, USA) and a serial dilution of the compounds 9–16 was made directly on the plate. The final drug concentrations tested were between 0.01 and 2.0 µl/ml. Plates were covered and sealed with parafilm and incubated at 37°C for 5 days. After this time, 25 µl of a freshly prepared 1:1 mixture of Alamar Blue (Accumed International, Westlake, Ohio, USA) reagent and 10% tween 80 was added to the plate and incubated for 24 h. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC (Minimal Inhibition Concentration) is defined as the lowest drug concentration, which prevents the colour change from blue to pink.

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