Synthesis of Thiol-Containing Analogues of Puromycin and a Study of their Interaction with *N*-Acetylphenylalanyl-Transfer Ribonucleic Acid on Ribosomes to Form Thioesters

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1. The thiol-containing analogue of puromycin, 6-dimethylamino-9-[1'-[3'-(2''-mercapto-3''-phenylpropionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XVII) in which the primary amino group of the antibiotic is replaced with a thiol grouping, was synthesized chemically (compound XVII is abbreviated to thiopuromycin). 2. Thiopuromycin (XVII) was found to be active in releasing *N*-[3 H]acetylphenylalanine from its tRNA carrier as the thioester, *N*-acetylphenylalanylthiopuromycin (XIX) in the *Escherichia coli* ribosomal system. The reaction product (XIX) was synthesized chemically from thiopuromycin and *N*-acetylphenylalanine and found to be stable to hydrolysis in the standard incubation medium at pH 7.6. DL-Phenyl-lactylpuromycin (XXI), the hydroxy analogue of puromycin, was also synthesized chemically and shown to release *N*-acetylphenylalanine from its tRNA carrier in the *E. coli* ribosomal system, thus confirming the previous results of Fahnestock *et al.* [*Biochemistry* (1970) 9, 2477–2483]. 3. In marked contrast with the results obtained in the *E. coli* system, both thiopuromycin (XVII) and hydroxypuromycin (XXI) were found to be inactive in releasing *N*-acetylphenylalanine from its tRNA carrier in the rat liver ribosomal system.

The formation of peptide bonds in protein synthesis is catalysed by the peptidyltransferase enzyme of the 50S ribosomal subunit (Monro, 1967). The reaction involves an activated aminoacyl- or peptidyl-tRNA to give an acid amide. The reaction between puromycin and *N*-acetylphenylalanyl-tRNA (Scheme 1) is often used as a model for studying the peptidyltransferase reaction. In attempts to further elucidate and characterize the properties of the peptidyltransferase, Rich's group (Fahnestock *et al.*, 1970; Fahnestock & Rich, 1971) have synthesized a derivative of puromycin in which the primary amino group of the molecule is replaced by a hydroxy group. They made the interesting observation that the hydroxypuromycin derivative reacted with Met-tRNA in the *Escherichia coli* ribosomal system to give fMet-hydroxypuromycin in which the amino acid is linked via an ester linkage to the hydroxypuromycin (Scheme 1). As mentioned by Rich's group (Fahnestock *et al.*, 1970; Fahnestock & Rich, 1971), many proteinase enzymes that normally break peptide or acid amide bonds can also function as esterases (Riordan & Vallee, 1963, 1964), and these facts may have a bearing on our attempts to understand the mechanism of the ribosomal peptidyltransferase.

In furthering these ideas, we have been interested in the possibility that in a model system, thiol

derivatives of puromycin might react with aminoacyl- or peptidyl-tRNA on the ribosome to give thioesters, thus paralleling the results on ester formation (Scheme 1).

In the present paper we describe the synthesis of certain thiol-containing derivatives of puromycin and further demonstrate that the derivative that contains an aromatic benzene ring system is active in releasing *N*-acetylphenylalanine from its tRNA carrier in the *E. coli* ribosomal system. Thiopuromycin was found to be inactive in the rat liver ribosomal system.

Materials and Methods

Chemicals and enzymes

Cellulose MN 300 was supplied by Machery, Nagel and Co., Düren, Germany. Cellulose, Whatman standard grade, was obtained from W. and R. Balston, Maidstone, Kent, U.K. Puromycin dihydrochloride, puromycin aminonucleoside and GTP (disodium salt) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Dicyclohexylcarbodiimide was supplied by Koch-Light Laboratories, Colnbrook, Bucks., U.K. Monochloroacetic acid and aniline were obtained from May and Baker, Dagenham, Essex, U.K. 1,1,3,3,3-Tetramethylbutylamine was supplied by K & K Laboratories, Plain-

Scheme 1. Reaction of aminoacyl-tRNA with (a) puromycin to give an acid amide, (b) hydroxypuromycin to give an ester and (c) thiopuromycin to give a thioester

All three reactions are catalysed by the peptidyltransferase of the *E. coli* ribosome. B, 6-Dimethylaminopurine.

view, Calif., U.S.A. Quinine sulphate was obtained from Geddes Ltd., Salisbury, Rhodesia. Triethylamine was supplied by E. Merck A.C., Darmstadt, Germany. [^3H]Puromycin aminonucleoside (2Ci/mmol) and L-[^3H]phenylalanine (29Ci/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of analytical grade. Solvents were dried and redistilled. Before use they were stored over pellets of molecular-sieve compound 3A.

Preparation of subcellular fractions

Polyribosomes were prepared from homogenates of rat liver in medium A [containing (final concentrations): sucrose (0.25M), MgCl_2 (5mM), KCl (25mM) and Tris-HCl buffer, pH 7.6 (50mM)] by the method of Wettstein *et al.* (1963) as described for the Spinco no. 30 rotor by Hawtreay & Nourse (1966). They were finally suspended in medium A and frozen (-15°C).

Washed rat liver polyribosomes

These were prepared by a modification of the method of Siler & Moldave (1969) as described by Ariatti & Hawtreay (1975).

Translocation (G factor)

This was prepared from a sample of rat liver pH 5 supernatant as described by Felicetti & Lipmann (1968) and finally frozen in small portions at -15°C .

N-Acetyl[^3H]phenylalanyl-tRNA

This was prepared as described by Ariatti & Hawtreay (1975). It gave an E_{260}/E_{280} ratio of 1.80 and 38×10^4 c.p.m./100 μg of tRNA.

E. coli ribosomes

E. coli ribosomes were prepared in medium A essentially by the method of Tissieres *et al.* (1959). They were suspended in a buffer containing

NH₄Cl (0.5M), Tris-HCl, pH 7.6 (50mM), magnesium acetate (10mM) and dithiothreitol (1mM) and washed by centrifugation through a discontinuous gradient containing 0.5M-sucrose in the above buffer. Centrifugation was at 105000g for 3h in the no. 40 rotor. They were finally suspended in medium A and stored frozen at -15°C.

Measurement of radioactivity

In certain experiments, reactions were stopped with an equal volume of ice-cold 10% (w/v) trichloroacetic acid and the samples transferred to Whatman GF/C filters. Each filter was then washed with 25ml of ice-cold 5% (w/v) trichloroacetic acid, dried and counted for radioactivity in toluene containing 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene. Radioactivity in ethyl acetate extracts was counted in Bray's (1960) solution.

Cellulose chromatography

The following solvent systems were used: A, 2-methylbutan-2-ol-formic acid-water (10:1:2, by vol.); B, 2-methylbutan-2-ol-formic acid-water (3:2:1, by vol.); C, 2-methylbutan-2-ol-formic acid-water (50:1:10, by vol.).

Thin-layer cellulose chromatography. For qualitative analysis, chromatography was carried out with small glass slides (7.5cm×2.5cm) plated with cellulose (MN 300), 0.2mm thick, in solvent A.

For preparative chromatography, large plates (20cm×10cm) were used. Cellulose (MN 300) was coated at 1mm thickness. Before use, plates were developed with solvent B to remove a contaminating yellow oil which was present in the cellulose powder. Plates were then dried before application of the samples. Development was carried out with solvent A. All chromatograms were examined under u.v. light (254nm). Thiols were detected by spraying with a filtered solution containing 1.5g of sodium nitroprusside in 5ml of 2M-HCl plus 95ml of methanol and 10ml of aq. NH₃ (sp.gr. 0.88). This spray was later found to be very unreliable with many puromycin analogues, and was replaced by one containing 0.2g of *p*-nitrosodimethylaniline and 1g of *p*-dimethylaminobenzaldehyde in 100ml of ethanol. After being sprayed, the plates were heated to 100–110°C for 5min.

Cellulose column chromatography. Cellulose (Whatman chromatography grade) was extracted with ethanol in a Soxhlet extractor for 48h before use. Columns were packed with washed cellulose (dimensions of columns given in individual experiments) and washed with solvent B until no deposit was left when sample of the eluate was evaporated to dryness. Solvent A was used in many chromatographic runs. It was found later that solvent C gave

a much better separation of the compounds under study. This solvent was therefore used in preference to solvent A.

Chemical syntheses

Bromoacetic acid (I). This was prepared under standard conditions from acetic acid, bromine and phosphorus trichloride. It was finally recrystallized twice from *n*-hexane, m.p. 49.5–50°C. Mass spectrometry gave molecular ions, *m/e* of 138 and 140 (*M*⁺) (theoretical *m/e* 138 and 140).

*Potassium *o*-ethyl dithiocarbonate (II).* This was prepared by the method of Vogel (1964) and recrystallized from ethanol.

Potassium thiolacetate (III). Thiolacetic acid (3.0ml) and solid pellets of KOH (2.2g) were mixed with ethanol (5.0ml) and shaken for 1h at room temperature (22°C). Ethanol was then removed under reduced pressure and the required material obtained directly from the residue by two extractions with ethyl acetate containing a little ethanol, followed by recrystallization from the same system. The material crystallized as long colourless needles.

1,1,3,3-Tetramethylbutylamine thiolacetate (IV). To 3.0ml (30mmol) of thiolacetic acid was added an excess of 1,1,3,3-tetramethylbutylamine (14.5g, 70mmol) at room temperature. No solvent was added. After 30min the resulting pale-yellow solid was extracted with hot ethyl acetate. On cooling, the desired material separated. It was twice recrystallized from ethyl acetate to give white fibrous crystals, m.p. 74°C (decomp.) (Found: C, 58.2; H, 11.3; N, 6.9; S, 15.0; C₁₀H₂₃ONS requires C, 58.4; H, 11.3; N, 6.8; S, 15.6%).

2-Thioacetylacetic acid (V). Equivalent amounts of monobromoacetic acid (250mg, 1.8mmol) and potassium thiolacetate (206mg, 1.8mmol) were mixed together in anhydrous acetone (10ml), and the mixture was refluxed for 15min. The precipitated KBr was removed by filtration. Acetone was removed under reduced pressure, and the resulting oil dissolved in ether. The ether extract was washed successively with dilute HCl and water, and then dried over anhydrous Na₂SO₄. After evaporation of the ether, the product was obtained as a clear colourless oil (Found: C, 35.8; H, 4.6; S, 23.6; C₄H₆O₃S requires C, 35.8; H, 4.5; S, 23.8%).

*2-(*O*-Ethyl dithiocarbonate)ethanoic acid (VI).* This was prepared from chloroacetic acid and potassium *O*-ethyl dithiocarbonate by the method of Djerassi *et al.* (1955). It was recrystallized from light petroleum (b.p. 80–100°C), m.p. 55–55.5°C; *m/e* 180 (*M*⁺), theoretical *m/e* 180 (Found: C, 33.3; H, 4.4; S, 35.5; C₅H₈O₃S₂ requires C, 33.5; H, 4.4; S, 35.4%).

DL-2-Bromo-3-phenylpropionic acid (VII). This was prepared by the method of Marvel (1955) by using superdry ethanol. The product was distilled under a pressure of 150–240Pa, b.p. 146–148°C.

Scheme 2. Synthesis of 2-thioacetyl-3-phenylpropionic acid (VIII) and 2-(O-ethyl dithiocarbonate)-3-phenylpropionic acid (IX)

The lower part of the Scheme shows neighbouring-group participation of the carboxyl group of 2-bromo-3-phenylpropionic acid (VII) in its reactions with salts of thioacetates or xanthates, thus giving overall retention of configuration.

Yield was 61% of theoretical. The racemic acid was resolved via formation of the quinine salt, as described by Fischer & Carl (1906); L(+)-acid, $[\alpha]_{\text{D}}^{25} +4.26^\circ$; D(-) acid, $[\alpha]_{\text{D}}^{25} -5.85^\circ$.

D-2-Thioacetyl-3-phenylpropionic acid (VIII, Scheme 2). D(-)-2-Bromo-3-phenylpropionic acid (VII) (2.3 g) was mixed with potassium thioacetate (1.25 g) in acetone (70 ml) and refluxed for 8 min. The mixture was left in ice overnight. The precipitated KBr was removed by filtration, and the acetone removed under a stream of N_2 . The resulting oil was dissolved in ether and was washed with 5×25 ml of water. Water was removed with Na_2SO_4 and the solvent removed under N_2 to give a thick yellow oil; yield 1.5 g. The oil crystallized at low temperature in the 'deep-freeze', m/e 224 (M^+), $[\alpha]_{\text{D}}^{25.2} +43^\circ$.

D-2-(O-Ethyl dithiocarbonate)-3-phenylpropionic

acid (IX, Scheme 2). This was prepared from D(-)-2-bromo-3-phenylpropionic acid (VII) and potassium O-ethyl dithiocarbonate by the method used for the ethanoic acid dithiocarbonate. Recrystallization from cyclohexane yielded shining plates, m.p. $92-93^\circ\text{C}$; $[\alpha]_{\text{D}}^{22.3} +41^\circ$ (Found: C, 54.48; H, 5.27; S, 23.46; $\text{C}_{12}\text{H}_{14}\text{S}_2\text{O}_3$ requires C, 53.51; H, 5.22; S, 23.72%).

L-2-(O-Ethyl dithiocarbonate)-3-phenylpropionic acid (IX). This was prepared as described above from L(+)-2-bromo-3-phenylpropionic acid (VII) and potassium O-ethyl dithiocarbonate. Recrystallization was from cyclohexane. Melting point of a portion was 83°C and the rest melted at $88-90^\circ\text{C}$. The L-acid was therefore further resolved via its quinine salt through four repeated crystallizations. The final free acid was recrystallized from light petroleum (b.p. $80-100^\circ\text{C}$), m.p. $93-94^\circ\text{C}$; $[\alpha]_{\text{D}}^{22.2} -49^\circ$.

6-Dimethylamino-9-[1'-[3'-(2"-O-ethyl dithiocarbonate-acetamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XI, Scheme 3). Puromycin aminonucleoside (X) (50mg, 0.17mmol) was dissolved in 1.0ml of dimethylformamide with heating. To this solution, 2-(O-ethyl dithiocarbonate)ethanoic acid (VI) (79mg; 0.14mmol) in 0.5ml of dimethylformamide was added, followed by a solution of *NN*-dicyclohexylcarbodi-imide (49mg, 0.24mmol) in 0.3ml of the same solvent. The reaction mixture was left for 20h at room temperature. Thereafter dicyclohexylurea was removed by filtration and the solvent removed under reduced pressure. The oily crystalline residue was triturated with dry ether and left for several hours. The procedure induced most of the desired product to solidify. Decantation of the ether removed unchanged acid. The product was washed with further portions of ether. The solid residue was dissolved in hot dioxan. On cooling a further quantity of dicyclohexylurea crystallized out, which was removed by filtration. The dioxan solution was then evaporated to dryness under reduced pressure, and the solid residue dissolved in a few ml of solvent A, which was then placed on a cellulose column (36cm $\times$ 1.8cm) and eluted with solvent A. Fractions (5ml) were collected. The desired product was found in fractions 16–20. Solvent was removed under reduced pressure to give a solid, which was washed with dry ether and then recrystallized from ethanol to give rosette-like

crystals (63.5mg, 82%), m.p. 175–180°C (decomp.) (Found: C, 44.7; H, 5.3; N, 18.9; $C_{17}H_{24}O_5N_6S_2$ requires C, 44.7; H, 5.3; N, 18.4%). $\lambda_{\max}$. (dioxan) 278.5nm (log ϵ 4.65). $\nu_{\max}$. (KBr) 1660cm $^{-1}$ (C=O amide I); 1050cm $^{-1}$ (C=S).

6-Dimethylamino-9-[1'-[3'-(2"-bromoacetamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XII, Scheme 3). Bromoacetic acid (112mg, 0.81mmol) dissolved in 0.3ml of dimethylformamide was added to puromycin aminonucleoside (X) (100mg, 0.34mmol) in 2ml of dimethylformamide. This solution was warmed to 45°C, and a solution of *NN*-dicyclohexylcarbodi-imide (116mg, 0.56mmol) in 0.5ml of dimethylformamide added. The mixture was then left for 20h at room temperature. Dicyclohexylurea was removed by filtration and the clear solution was evaporated to dryness under reduced pressure. Ether extraction and hot dioxan treatment was carried out on the residue as described above for the dithiocarbonate-acetamido derivative. The crude product was dissolved in a few ml of solvent A and chromatographed on a cellulose column (40cm $\times$ 2cm) and eluted with solvent A. Fractions (5ml) were collected, and the desired product appeared in fractions 21–24. The solvent was removed under reduced pressure and the residue recrystallized from ethanol (76.5mg, 54%), m.p. 195–197°C; m/e 414 and 416 (M^+). $\lambda_{\max}$. (dioxan) 276nm (log ϵ 4.45). $\nu_{\max}$. (KBr) 1675cm $^{-1}$ (C=O amide I).

Scheme 3. Synthesis of glycylythiopuromycin (XIV)

Scheme 4. Synthesis of thiopuromycin (XVII)

6-Dimethylamino-9-[1'-[3'-(2"-thioacetylacetamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XIII, Scheme 3). This was prepared by two methods. (a) From 6-dimethylamino-9-[1'-[3'-(2"-bromoacetamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XII). A solution of 50 mg (0.12 mmol) of the bromoacetamido derivative in 5.5 ml of dioxan-methanol (95:5, v/v) was added to 69 mg (0.33 mmol) of 1,1,3,3-tetramethylbutylamine thiolacetate (IV) in 1.1 ml of the same solvent. The mixture was left at room temperature for 19 h. The solvent was removed under reduced pressure and the resulting residue was washed with 3 $\times$ 5 ml of acetone-ether (1:1, v/v). The product was recrystallized from ethanol to give rosettes (23 mg, 49%); m.p. 182–184°C; m/e 410, 452 and 494 (M^+) (see the Discussion section). λ_{max} , 276.5 nm ($\log \epsilon$ 4.51). ν_{max} , (KBr) 1680 cm^{-1} (C=O thioester) 1640 cm^{-1} (C=O amide I). (b) Directly from puromycin aminonucleoside (X). To a solution of puromycin aminonucleoside (52 mg, 0.17 mmol) in 1.2 ml of dimethylformamide, 2-thioacetylacetic acid (V) (55 mg, 0.41 mmol) in 0.2 ml of dimethylformamide was added. The mixture was warmed to 45°C and *NN*-dicyclohexylcarbodi-imide (53 mg, 0.26 mmol) in 0.2 ml of dimethylformamide

was added, and the reaction mixture left at room temperature overnight. Partial purification of the product was carried out as above for the dithiocarbonate derivative. This material was dissolved in a few ml of solvent C and placed on a cellulose column (41 cm $\times$ 2 cm) and eluted with solvent C. Fractions (5 ml) were collected and the desired product was recovered from fractions 23–27 by evaporation under reduced pressure. The product was recrystallized from ethanol (46.8 mg, 64%), m.p. 181–184°C, m/e 410, 452 and 494 (M^+) (see the Discussion section). Optical data were as above.

6-Dimethylamino-9-[1'-[3'-(2"-mercaptoacetamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XIV, Scheme 3). This was prepared by two methods.

(a) Anhydrous NH_3 gas was bubbled through a solution of 6-dimethylamino-9-[1'-[3'-(2"-thioacetyl-amido)-3'-deoxy- β -D-ribofuranosyl]]purine (XIII) (40 mg, 0.097 mmol) in 4 ml of anhydrous methanol at room temperature for 10 min. The solvent was removed under a stream of N_2 and finally with reduced pressure. The resulting white powder was washed twice with small portions of dry ether, and recrystallized from ethanol as fine colourless needles (33.5 mg,

93%), m.p. 208.5–209.5°C, m/e 369 (M^+). $\lambda_{\max}$. (dioxan) 275.5 nm ($\log \epsilon$ 4.50). $\nu_{\max}$. (KBr) 1640 cm^{-1} (C=O amide I).

(b) Via 6-dimethylamino-9-[1'-[3'-(2"-O-ethyl dithiocarbonate-acetamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XI). The dithiocarbonate-acetamido derivative (10 mg, 0.02 mmol) was dissolved in 2 ml of approx. 16M-NH₃ in methanol at -20°C and left for 45 min at this temperature. After removal of the solvent under a stream of N₂ below 0°C, the material was dissolved in methanol-chloroform (1:9, v/v) and put on to three thin-layer plates (20 cm × 10 cm) of Silica gel G-254 and developed with methanol-chloroform (1:9, v/v). The appropriate band was removed and the product eluted with the same solvent. After removal of the solvent, the product when placed on a thin-layer silica-gel G-254 plate and developed in methanol-chloroform (1:9, v/v) proved to contain two components. These were separated on silica-gel plates in methanol-chloroform (1:12, v/v). The material obtained proved to be identical with that obtained via the thioacetyl acetamide derivative (2.3 mg, 28.5%).

6-Dimethylamino-9-[1'-[3'-(D-2"-thioacetyl-3"-phenylpropionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XV, Scheme 4). A solution of D-2-thioacetyl-3-phenylpropionic acid (VIII) (224 mg, 1.0 mmol) in 0.6 ml of dimethylformamide was added to puromycin aminonucleoside (X) (139 mg, 0.47 mmol) in 3.5 ml of dimethylformamide. This mixture was warmed to 45°C and *NN*-dicyclohexylcarbodi-imide (167 mg, 0.82 mmol) in 0.6 ml of dimethylformamide was added and then left overnight at room temperature. The product was partially purified as described for the dithiocarbonate-acetamido derivative. The partially purified material was dissolved in a few ml of solvent C, placed on a cellulose column (32.5 cm × 2.0 cm) and eluted with solvent C. Fractions (5 ml) were collected and the desired product was recovered from fractions 13–19 by removing the solvent under reduced pressure (220 mg). The yellowish solid obtained was washed with dry ether and then recrystallized from ethanol (169 mg 71%), m.p. 193–196°C, m/e 500 (M^+). $\nu_{\max}$. (KBr) 1693 cm^{-1} (C=O thioester); 1650 cm^{-1} (C=O amide I).

6-Dimethylamino-9-[1'-[3'-(2"-O-ethyl dithiocarbonate-3"-phenylpropionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XVI, Scheme 4). The usual procedure was followed in the synthesis of both the D and L isomers. We describe here the synthesis of the L isomer only. To 85 mg (0.29 mmol) of puromycin aminonucleoside (X) and 154 mg (0.56 mmol) of L-2-(O-ethyl dithiocarbonate)-3-phenylpropionic acid (IX) in 2.2 ml of dimethylformamide was added 87 mg (0.42 mmol) of *NN*-dicyclohexylcarbodi-imide in 0.2 ml of dimethylformamide. The partially purified product was dissolved in a few ml of solvent C and placed on a cellulose column (32.5 cm × 2.0 cm)

and eluted with solvent C. Fractions (5 ml) were collected and the desired product was obtained from fractions 13–15 after evaporation of the solvent under reduced pressure. Recrystallization from ethanol (105 mg, 66%); m.p. 184°C (decomp.) (Found: C, 53.03; H, 5.69; N, 15.08; C₂₄H₃₀N₆S₂ requires C, 52.73; H, 5.53; N, 15.37%).

6-Dimethylamino-9-[1'-[3'-(2"-mercapto-3"-phenylpropionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XVII, Scheme 4). This was prepared by two methods.

(a) Via compound (XVI). Both the D and L isomers were synthesized via the same procedure. The synthesis of the D isomer (XVII) is described here. D isomer of compound XVI (185 mg) was treated for 30 min with 10 ml of NH₃-methanol (methanol saturated with anhydrous NH₃ gas at -10°C). The reaction was started at -10°C and left to warm to room temperature. (It was later found that greater efficiency was obtained by using approx. 16M-NH₃ in methanol, prepared by dissolving NH₃ gas in methanol at -22°C and running the reaction at -20°C for 30–45 min.) The reaction mixture was dried under a stream of N₂ in the cold. The solid material was dissolved in methanol-chloroform (1:9, v/v) and placed on fifteen thin-layer silica-gel G-254 plates (20 cm × 10 cm). The plates were developed in methanol-chloroform (1:19, v/v). The appropriate bands were removed and eluted with methanol-chloroform (1:9, v/v). After filtration, the solvent was removed from the filtrate under reduced pressure. The resulting white material was recrystallized from methanol to give the D-isomer (39 mg, 21%), m.p. 200–204°C, m/e 458 (M^+). The L isomer had m.p. 216–219°C, m/e 458 (M^+).

(b) Via 6-dimethylamino-9-[1'-[3'-(2"-D-thioacetyl-3"-phenylpropionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XV). D isomer of compound (XV) (25 mg, 0.05 mmol) was dissolved in 4.5 ml of 16M-NH₃ in methanol at -20°C and placed in the 'deep-freeze' at this temperature for 1 h. The solvent and NH₃ were removed under a stream of N₂, the temperature being kept below 0°C. The solid material obtained was dissolved in a small amount of methanol-chloroform (1:9, v/v), put on to six silica-gel G-254 plates (20 cm × 10 cm) and developed with methanol-chloroform (1:16, v/v). The appropriate bands (see the Discussion section) were removed and eluted with methanol-chloroform (1:9, v/v). After filtration, the solvent was removed from the filtrate under reduced pressure, and a white crystalline material (11.5 mg, 50%) was obtained, which proved to be identical with the D-isomer (D isomer of compound XVII) prepared via the xanthate (see method a above); m.p. 202–204°C; m/e 458 (M^+). The i.r. spectra were identical for the two compounds prepared via the different methods.

N-Acetyl-L-[³H]phenylalanine. This was prepared by a standard procedure from L-[³H]phenylalanine

Scheme 5. Synthesis of *N*-acetylphenylalanylthiopuromycin (XIX) Scheme 6. Synthesis of *DL*-hydroxypuromycin (XXI)

(plus unlabelled carrier). The final product was recrystallized from water, m.p. 167–169°C, radioactivity 1.44×10^5 c.p.m./ μ mol.

N-Hydroxysuccinimide ester of *N*-acetyl-L-[3 H]-phenylalanine (XVIII, Scheme 5). The radioactive ester was prepared as described by Ariatti & Hawtre (1975). The labelled product was recrystallized from propan-2-ol to give rosettes, m.p. 143–146°C, radioactivity 1.40×10^5 c.p.m./ μ mol.

6-Di[3 H]methylamino-9-[1'-[3'-(2"-thio-{2"-acetamido-3"-phenylpropionyl}-3"-phenylpropionamido)-3'-deoxy- β -D-ribofuranosyl]purine (XIX, Scheme 5). A mixture of 8 mg (0.017 mmol) of thiopuromycin (XVII) and 9 mg (0.025 mmol) of the *N*-hydroxysuccinimide derivative of *N*-acetyl-L-[3 H]-phenylalanine (XVIII) was dried thoroughly under vacuum. The mixture was then redissolved in 0.3 ml of triethylamine-dimethylformamide (1:9, v/v) and left to react at room temperature for 70 min. The reaction mixture was dried under vacuum, the residue dissolved in chloroform and subjected to t.l.c. on silica-gel G-254 plates (20 cm $\times$ 20 cm) in methanol-chloroform (3:50, v/v) as solvent. The

appropriate band was eluted with the same solvent combination. After being dried, the residue was recrystallized from ethanol. The crystalline material was finally washed with dry ether (5.4 mg, 48%), m.p. 191–193°C. $\nu_{\max}$. (KBr) 1675 cm^{-1} (C=O thioester); 1655 cm^{-1} (C=O amide I). Radioactivity 1.2×10^5 c.p.m./ μ mol (Found: C, 59.2; H, 5.8; N, 14.8; S, 4.8; $\text{C}_{32}\text{H}_{37}\text{N}_7\text{SO}_6$ requires C, 59.3; H, 5.8; N, 14.8; S, 4.8%).

N-Hydroxysuccinimide ester of *DL*-2-hydroxy-3-phenylpropionic acid (XX, Scheme 6). To a mixture of 0.83 g (4.9 mmol) of *DL*-2-hydroxy-3-phenylpropionic acid and 0.58 g (5.0 mmol) of *N*-hydroxysuccinimide in 6.0 ml of tetrahydrofuran was added 1.5 g (7.3 mmol) of *NN*-dicyclohexylcarbodi-imide in 4.0 ml of tetrahydrofuran. After 2 h at room temperature, dicyclohexylurea was removed by filtration and the solvent removed under reduced pressure. The resulting oil was taken up in ethyl acetate and left overnight. More urea was then removed by filtration and the solution concentrated to an oil under vacuum.

6-Dimethylamino-9-[1'-[3'-(2"-hydroxy-3"-phenylpro-

pionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XXI, Scheme 6). Puromycin aminonucleoside (X) (39mg, 0.13mmol) and *N*-hydroxysuccinimide ester of DL-2-hydroxy-3-phenylpropionic acid (74mg, 0.28mmol) (XX) were dissolved in 0.7ml of pyridine. Water (0.3ml) was added and the reaction allowed to proceed at room temperature for 4h. The mixture was filtered to remove traces of urea and then concentrated to dryness under vacuum. The solid residue was washed with dry ether and then purified by t.l.c. on silica-gel G-254 plates (20cm $\times$ 20cm) in methanol-chloroform (6:94, v/v) as solvent. The appropriate band was eluted with the same solvent. The extract was filtered and taken to dryness. Recrystallization from ethanol (27.3mg, 47%), m.p. 218–221°C, *m/e* 442 (M^+). $\nu_{\max}$. (KBr) 1650cm $^{-1}$ (C=O amide I).

6-Di[3H]methylamino-9-{1'-[3'-(2"-L-mercapto-3"-phenylpropionamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XVII). This was prepared by the same method used for the synthesis of the unlabelled compound. Reactants were [3H]puromycin aminonucleoside (1mCi, 2.0Ci/mmol), unlabelled puromycin aminonucleoside (8.5mg, 0.029mmol), L-2-(O-ethyl dithiocarbonate)-3-phenylpropionic acid (IX) (18mg, 0.067mmol) and *NN*-dicyclohexylcarbodi-imide (13mg, 0.064mmol). After the usual work-up the yield was 3.1mg and radioactivity was 1.24×10^5 c.p.m./ μ mol.

Results and Discussion

In attempts to synthesize thiol-containing derivatives of puromycin (the phenylalanyl analogue), we originally decided to modify directly the primary amino group of puromycin with a view to eventually replacing this function with a thiol grouping. However, attempts in this direction led to deep-seated changes in the molecule and were therefore abandoned.

The simplest approach to obtaining the required substance (compound XVII, Scheme 4) appeared to be a method of synthesis in which the required protected phenylalanyl thiol-containing portion of the molecule (compounds VIII and IX of Scheme 2) was first made and then coupled to puromycin aminonucleoside (compound X, Scheme 4), followed by generation of the free thiol group. A similar approach was envisaged for the thiol-containing glycyl derivative of puromycin (compound XIV, Scheme 3), and this was a far more simple compound to theoretically prepare, its synthesis was attempted first.

The carbodi-imide coupling approach was followed (Scheme 3) in attaching 2-thioacetylacetic acid (V) to puromycin aminonucleoside (X) thus giving the intermediate (compound XIII), which was deacetylated with methanolic NH_3 to the required compound (XIV). A similar series of reactions with 2-(O-ethyl

dithiocarbonate)ethanoic acid (VI) gave the same final compound (XIV) via the intermediate compound (XI).

Having shown that the above coupling procedures were satisfactory, we turned our attention to the synthesis of the thiol-containing phenylalanylpuromycin derivative (compound XVII, Scheme 4).

As a first stage in the synthesis, either the D- or L-2-bromo-3-phenylpropionic acid (compound VII, Scheme 2) was treated with potassium thioacetate or potassium O-ethyl dithiocarbonate to give respectively the thioacetate (VIII) or xanthate (IX) (Schemes 2 and 4). We assume that in both reactions overall retention of configuration ensues owing to neighbouring-group participation of the carboxyl group of compound (VII) under neutral conditions (see explanation in the legend to Scheme 2). There is considerable evidence in the literature to support this assumption (Cram & Hammond, 1959). Further, our biochemical results support this conclusion.

The second stage of the synthesis involved coupling of the intermediate thioester (compound VIII) with puromycin aminonucleoside (X) in the presence of *NN*-dicyclohexylcarbodi-imide to give compound XV which was deacetylated with methanolic NH_3 to give the desired compound (XVII, Scheme 4). A similar series of reactions with the dithiocarbonate ester (compound IX) gave an identical final product (XVII, Scheme 4).

The mass spectra of 6-dimethylamino-9-{1'-[3'-(2"-thioacetylacetamido)-3'-deoxy- β -D-ribofuranosyl]]purine (XIII) showed minor peaks of $M+42$ and $M+84$. These were due to trace amounts of 2'- and 5'-O-acetylated material. The amount was small since the i.r. spectra did not contain any adsorption peaks attributable to an ester carbonyl.

The synthesis of *N*-acetylphenylalanylthiopuromycin is shown in Scheme 5, and the synthesis of DL-hydroxy puromycin is shown in Scheme 6.

Biochemical results

To assay the activity of thiopuromycin as a possible releasing agent for peptides on the ribosome, we used the well-known standard procedure involving the reaction between ribosomes carrying *N*-acetyl[3H]phenylalanyl-tRNA and the puromycin derivative under study. Ethyl acetate extraction at pH 7.6 was used to isolate the released product of the reaction.

Results of the interaction of *N*-acetyl[3H]phenylalanyl-tRNA with thiopuromycin in the *E. coli* ribosomal system are shown in Table 1. It is seen that L-thiopuromycin (XVII, Scheme 4) releases *N*-acetyl[3H]phenylalanine into an ethyl acetate-extractable form, and that the compound is approx. 50% as active as puromycin at the same concentration. D-Thiopuromycin also brings about release but it is only approx. 7% as active as puromycin. The

Table 1. *Interaction between N-acetyl[³H]phenylalanyl-tRNA, puromycin, thiopuromycin and hydroxypuromycin in the E. coli ribosomal system*

Each reaction tube contained in a final volume of 0.5 ml: Tris-HCl buffer, pH 7.6 (50 mM); KCl (60 mM); MgCl₂ (20 mM); NH₄Cl (0.15 M); mercaptoethanol (1 mM); poly(U) (60 µg); N-acetyl[³H]phenylalanyl-tRNA (16 µg, 5.3×10^4 c.p.m.); washed ribosomes (0.6 mg). Incubations were carried out for 20 min at 37°C. Each reaction tube received 1.0 ml of 0.1 M-Tris-HCl (pH 7.6) and 3 ml of ethyl acetate. After mixing and centrifugation, portions (1 ml) of the upper phase were taken for radioactivity counting in Bray's (1960) solution. Each sample was corrected for radioactivity extracted by a blank determination. All reaction tubes contained 10% (v/v) dimethyl sulphoxide. The dimethyl sulphoxide was found in separate experiments to have no effect on the reactions studied. Glycylthiopuromycin was inactive in the above assay system

Assay system	N-Acetyl[³ H]phenylalanine extracted into ethyl acetate (c.p.m./ml)
Puromycin (1 mM)	1921
L-Thiopuromycin (1 mM)	922
D-Thiopuromycin (1 mM)	133
DL-Hydroxypuromycin (2 mM)	868

D-isomer is contaminated to the extent of approx. 10% with L isomer, and this may account for its observed activity. In separate experiments the K_m value for the reaction between L-thiopuromycin and N-acetyl[³H]phenylalanyl-tRNA in the *E. coli* ribosomal system was found to be 1.7×10^{-3} M. Fahnestock *et al.*, (1970) reported K_m values of 1.7×10^{-4} M and 1.0×10^{-3} M for puromycin and L-hydroxypuromycin respectively in the *E. coli* ribosomal system, the N-formylmethionine fragment reaction being used. It is noteworthy that Nathans & Neidle (1963) found D-phenylalanylpuromycin to be 20% as active as the L isomer in inhibiting protein synthesis in an *E. coli* system. DL-Hydroxypuromycin [previously synthesized but not chemically characterized by Rich's group (Fahnestock *et al.*, 1970)] was approx. 45% as active as puromycin in the release assay (Table 1). This latter result confirms the work of Fahnestock *et al.* (1970) in the *E. coli* system.

Having shown that L-thiopuromycin (XVII, Scheme 4) releases N-acetyl[³H]phenylalanine from tRNA in the *E. coli* system, it was considered important to determine whether the product of release was N-acetylphenylalanylthiopuromycin (thioester, Scheme 1) or not. N-Acetylphenylalanylthiopuromycin (compound XIX, Scheme 5) was therefore synthesized chemically. Fig. 1(a) shows that the radioactive compound obtained via ethyl acetate extraction in the reaction between L-thiopuromycin and N-acetyl[³H]phenylalanyl-tRNA moves with an

Fig. 1. *Reaction of L-thiopuromycin with N-acetyl[³H]-phenylalanyl-tRNA bound to E. coli ribosomes and an examination of the reaction products by t.l.c.*

Reaction mixtures were as for Table 1 and were incubated for 20 min at 37°C. Each reaction tube received 1 ml of 0.1 M-Tris-HCl (pH 7.6) and 3 ml of ethyl acetate. After mixing and centrifugation 2 ml of the upper phase was removed for further examination. The 2 ml of ethyl acetate extract was split into separate 1 ml samples and dried under N₂. One sample was treated with a few drops of 1 M-NaOH for 5 min. Both samples were then subjected to t.l.c. on silica-gel G-254 plates (10 cm × 20 cm) along with thiopuromycin and N-acetylphenylalanylthiopuromycin as markers in methanol-chloroform (1:19, v/v) as solvent. The positions of u.v.-absorbing spots were noted and the plate was divided into 0.5 cm-wide strips. The strips were cut out with a scalpel and each strip was extracted with methanol-chloroform (1:2, v/v). After centrifugation and filtration, extracts were placed in counting vials, evaporated to dryness and then counted for radioactivity in Bray's (1960) solution. (a) Thin-layer chromatogram of reaction products; (1), N-acetylphenylalanine; (2), N-acetylphenylalanine-thiopuromycin (XIX); (3), thiopuromycin. (b) Thin-layer chromatogram of reaction products subjected to alkaline hydrolysis; (1), sodium salt of N-acetylphenylalanine.

identical R_f value with that of synthesized N-acetylphenylalanylthiopuromycin on t.l.c., suggesting that the two compounds are identical. Further, brief

Table 2. Interaction between *N*-acetyl[³H]phenylalanyl-tRNA, puromycin, thiopuromycin and hydroxypuromycin in the rat liver ribosomal system

Each reaction tube contained the following in a final volume of 0.5ml: Tris-HCl, pH 7.6 (50mM); KCl (60mM); MgCl₂ (20mM); NH₄Cl (20mM); mercaptoethanol (1mM); GTP (potassium salt), pH 7.6 (0.5mM); poly(U) (50μg); *N*-acetyl[³H]phenylalanyl-tRNA (28μg, 10.7 × 10⁴c.p.m.); G factor (0.3mg of protein); washed ribosomes (0.8mg). Incubations were for 30min at 37°C. Each reaction tube in Expt. (a) received 1.0ml of 0.1M-Tris-HCl (pH 7.6) and 3ml of ethyl acetate and in Expt. (b) 1ml of 0.25M-potassium acetate (pH 5.5) plus 3ml of ethyl acetate. After mixing and centrifugation, portions (2ml) of the upper phase were counted for radioactivity in Bray's (1960) solution. Appropriate blank corrections were made for each reaction. All reaction tubes contained 10% (v/v) dimethyl sulphoxide. This solvent had no effect on the puromycin reaction (separate experiments).

System	<i>N</i> -Acetyl[³ H]phenylalanine extracted into ethyl acetate (c.p.m./2ml)
Expt. (a)	
Puromycin (1mM)	5141
L-Thiopuromycin (1mM)	295
D-Thiopuromycin (1mM)	105
DL-Hydroxypuromycin (2mM)	244
Expt. (b)	
Puromycin (1mM)	3489
L-Thiopuromycin (1mM)	— (below blank)
DL-Hydroxypuromycin (2mM)	— (below blank)

Table 3. Effect of washed rat liver ribosomes and G factor on the possible breakdown of *N*-acetyl[³H]phenylalanylthiopuromycin

Each reaction tube contained the following in a final volume of 0.25ml: Tris-HCl buffer, pH 7.6 (50mM); KCl (60mM); MgCl₂ (20mM); NH₄Cl (20mM); mercaptoethanol (1mM); GTP, pH 7.6, (0.5mM); poly(U) (25μg); *N*-Acetyl[³H]phenylalanylthiopuromycin (0.033μmol, 4000c.p.m.); washed ribosomes (0.4mg). All incubations were for 30min at 37°C. Reactions were stopped by the addition of 0.75ml of 0.1M-HCl followed by 3ml of diethyl ether. After mixing and centrifugation, portions (2ml) of the upper ether phase were counted for radioactivity in Bray's (1960) solution. All reaction tubes contained 10% (v/v) dimethyl sulphoxide.

System	<i>N</i> -Acetyl[³ H]phenylalanine released into ether phase (c.p.m./2ml)
Complete system	269
Complete system — ribosomes	225
Complete system — G factor	222
Complete system — ribosomes and G factor	204

Fig. 2. Reaction of [³H]puromycin and [³H]thiopuromycin with unlabelled peptide chains on rat liver polyribosomes

Reaction conditions were the same as those described for Table 2, except that NH₄Cl when present was at a concentration of 0.25M. [³H]Puromycin (1μCi); [³H]thiopuromycin (0.10μmol, 1.24 × 10³c.p.m.); polyribosomes (0.3mg). Incubations were at 37°C. Reactions were stopped by the addition of 0.50ml of 10% (w/v) trichloroacetic acid. The resulting precipitates were filtered on Whatman GF/C filters and washed with 20ml of 5% (w/v) trichloroacetic acid followed by 20ml of 96% ethanol. After drying, the filters were counted for radioactivity in toluene. All reaction tubes contained 10% (v/v) dimethyl sulphoxide. ●, [³H]Puromycin; ●, [³H]puromycin+0.25M-NH₄Cl; ○, [³H]thiopuromycin with and without 0.25M-NH₄Cl.

alkaline treatment of the ethyl acetate-extracted material followed by t.l.c. indicated that rapid breakdown of the thioester (XIX) had occurred to give thiopuromycin and *N*-acetylphenylalanine (Fig. 1b). It should be mentioned that the thioester (compound XIX) is stable to hydrolysis in the normal incubation medium at pH 7.6. These results show that L-thiopuromycin is capable of forming thioester in the *E. coli* ribosomal system.

Further experiments were carried out with rat liver ribosomes in the same assay system as for *E. coli* ribosomes. Results in Table 2 (Expt. a) indicate that both L-thiopuromycin and DL-hydroxypuromycin bring about very little release of *N*-acetyl[³H]phenylalanine from tRNA in the rat liver system (L-thiopuromycin, 7% and DL-hydroxypuromycin, 5% respectively of the release found with puromycin).

These results were obtained by using Tris-HCl (pH 7.6) and ethyl acetate. When potassium acetate (pH 5.5) buffer saturated with $(\text{NH}_4)_2\text{SO}_4$ and ethyl acetate were used in the extraction system (Table 2, Expt. b), no activity was found with either L-thiopuromycin or DL-hydroxy puromycin.

There existed the possibility in the rat liver system of either ribosome- or G factor-catalysed hydrolysis of the thioester product (XIX). For evaluating this possibility, N-acetyl[^3H]phenylalanylthiopuromycin (XIX) was synthesized chemically and then incubated with rat liver ribosomes or G factor. Results of this experiment are shown in Table 3. It is seen that the thioester (XIX) is stable under the normal incubation conditions (approx. 6% breakdown), therefore eliminating the suggestion above. The assay procedure depends on the insolubility of the thioester in diethyl ether under acidic conditions and the solubility of N-acetyl[^3H]phenylalanine (breakdown product) in ether.

A further series of experiments with the rat liver ribosomal system was carried out by using an assay (Skogerson & Moldave, 1968) that measured the direct coupling of labelled puromycin and labelled L-thiopuromycin (XVII) to unlabelled polypeptide chains on the ribosome (P site). Results in Fig. 2 show that L-[^3H]thiopuromycin does not couple with unlabelled peptides (acid-insoluble) on the rat liver ribosome even at high NH_4Cl concentrations.

The results of the present experiments show that the *E. coli* ribosomal system is indeed capable of forming, besides normal acid amides (peptide bonds), esters [as shown by Rich's group (Fahnestock *et al.*, 1970)] and thioesters. In contrast, rat liver ribosomes are incapable of forming either esters or thioesters. The reason for this difference is at present not known, but does indicate that the peptidyl synthetase enzyme complex of the *E. coli* ribosome is not specific for peptide-bond formation.

The mammalian rat liver ribosomal peptidyltransferase is, however, highly specific in this respect.

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