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The Synthesis and Biological Activity of 'Crippled Biotin'

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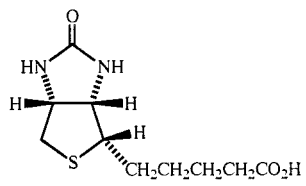
Abstract

(3aα,6β,6aα)-6-Methyltetrahydro-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one (2) was prepared as a crippled analogue of biotin. The key synthetic step involved hydrogenation of 6-methyl-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one on palladium to introduce the necessary all-*cis* configuration. Both compounds were weak inhibitors of the biotin-dependent wheat acetyl–CoA carboxylase compared to substrates or the potent herbicidal inhibitors of this enzyme, but were more potent than biotin or imidazolidone. Neither compound inhibited the biotin-dependent transcarboxylase component of bacterial acetyl–CoA carboxylase, nor did they significantly inhibit the growth of *Arabidopsis thaliana*.

Introduction

Fatty acid biosynthesis is almost identical in all organisms with acetyl–coenzyme A carboxylase (ACC) catalysing the first step—the ATP-dependent conversion of acetyl–CoA and carbon dioxide (derived from bicarbonate) into malonyl–CoA. In plants, malonyl–CoA is also the key intermediate in the biosynthesis of flavonoids, stilbenoids, naphthoquinones and other important compounds. (+)-Biotin (1) is a cofactor for ACC and its role is to transfer a carboxy group from a donor molecule at one site to an acceptor molecule at another site. It was recognized as an essential nutrient for living systems at the turn of the century, and its role as a cofactor for a family of enzymes that catalyse carboxylation, transcarboxylation and decarboxylation reactions in biological systems was discovered in 1960.[1]



(1)

[1] Wakil, S. J., Titchener, E. B., and Gibson, D. M., *Biochim. Biophys. Acta*, 1958, **29**, 225; Wakil, S. J., Gibson, D. M., *Biochim. Biophys. Acta*, 1960, **41**, 122.

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The only biologically active isomer of biotin (1), [3a*S*-(3aα,4β,6aα)]-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazole-4-pentanoic acid, has the all-*cis* configuration.

Biotin is attached to the enzyme by an amide linkage between its pentanoic acid side chain and the terminal amine of a lysine residue on the biotin carboxy carrier protein. This biotin–lysine unit is referred to as biocytin and provides an 'arm' approximately 14 Å long. This arm is thought to be able to swing back and forth (Fig. 1) carrying a carboxy group between the two sites where carboxylation and transfer occur.[2] It has been established that biotin carries the carboxy group on the nitrogen atom opposite the side chain.[3]

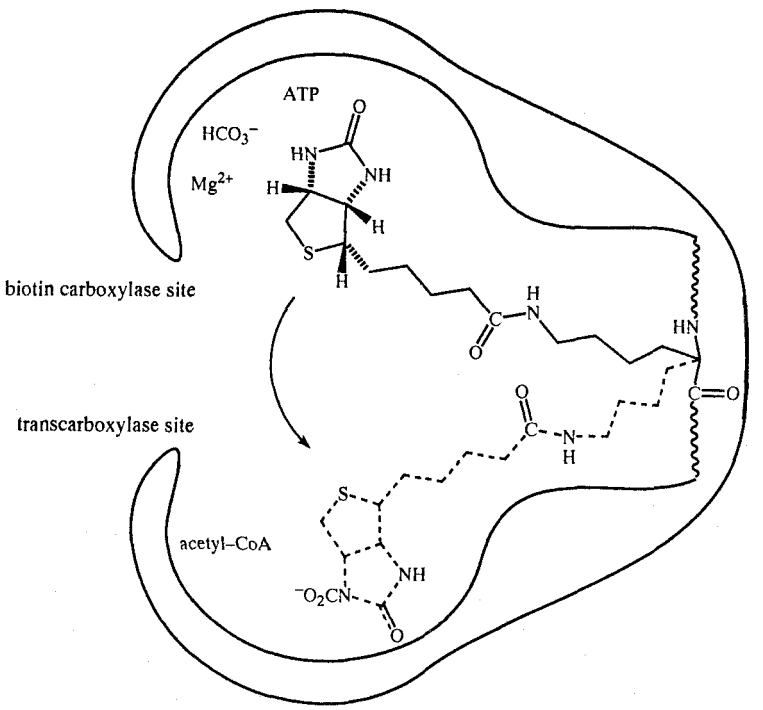


Fig. 1. Schematic diagram of acetyl–CoA carboxylase.

Important classes of herbicides (the aryloxyphenoxypropanonate and cyclohexanedione graminicides) are potent, reversible inhibitors of ACC and are believed to interfere with the carboxy-transfer site rather than the biotin carboxylation site of the enzyme.[4] To further understand this interaction, and to probe the enzyme's binding site, we wanted to design a biotin mimic that would be recognized by the enzyme but would be unable to function as a carboxy carrier. We use the term 'crippled biotin' to refer to a biotin analogue having the nitrogen atom, to

[2] Green, N. M., *Biochem. J.*, 1963, **89**, 585; Mildvan, A. S., Scrutton, M. C., and Utter, M. F., *J. Biol. Chem.*, 1966, **241**, 3488; Gregolin, C., Ryder, E., Warner, R. C., Kleinschmidt, A. K., Chang, H.-C., and Lane, M. D., *J. Biol. Chem.*, 1968, **243**, 4236; Northrop, D. B., and Wood, H. G., *J. Biol. Chem.*, 1969, **244**, 5801.

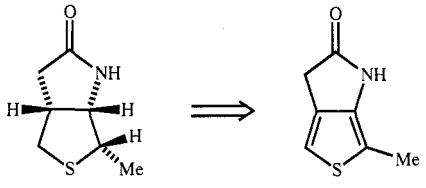
[3] Knowles, J. R., *Annu. Rev. Biochem.*, 1989, **58**, 195.

[4] Rendina, A. R., Craig-Kennard, A. C., Beaudoin, J. D., and Breen, M. K., *J. Agric. Food Chem.*, 1990, **38**, 1282; Rendina, A. R., and Felts, J. M., *Plant Physiol.*, 1988, **86**, 983.

which the carboxy group is attached during transfer (opposite the side chain), replaced by another group that is incapable of carboxy transfer.

Discussion

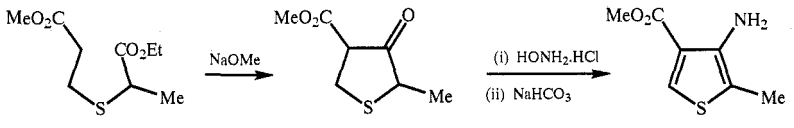
The tetrahydrothieno[3,4-*b*]pyrrol-2(3*H*)-one (2) was chosen as our synthetic target. In this compound the side chain of biotin is shortened to a methyl group to allow easy access into the enzyme site, and the opposite nitrogen atom is replaced by a methylene unit. Our key synthetic strategy to introduce the all-*cis* configuration was the palladium-catalysed hydrogenation of a thiophen precursor (3) (Scheme 1).



(2) (3) Scheme 1

Synthesis

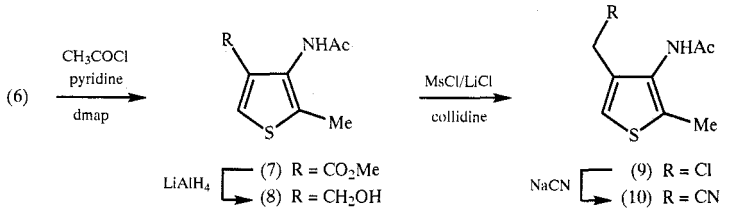
Condensation of ethyl 2-bromopropanoate and methyl 3-mercaptopropanoate gave ethyl 2-[2-(methoxycarbonyl)ethylthio]propanoate (4)[5] which underwent Dieckmann cyclization to give methyl 5-methyl-4-oxotetrahydrothiophen-3-carboxylate (5) in 70% yield. The keto ester (5) was a clear viscous oil which failed to give satisfactory elemental analysis but gave the expected accurate mass measurement. Both the ^{1}H and ^{13}C n.m.r. spectra of this compound were complex and indicated that it exists as an equilibrating mixture of *cis*, *trans* and enol isomers. An infrared absorption was observed at 1670 cm^{-1} which is consistent with an enolized β-keto ester.[6] Treatment of the ketone (5) with hydroxylamine hydrochloride in acetonitrile at reflux formed an oxime *in situ* which underwent Wolff–Semmler type aromatization[7] to give methyl 4-amino-5-methylthiophen-3-carboxylate (6) in 48% yield (Scheme 2). This conversion could also be carried out stepwise[8] by treating the ketone (5) with hydroxylamine hydrochloride and pyridine in ethanol to give the corresponding oxime (93% yield) which underwent aromatization to the thiophen (6) in 43% yield by treatment with hydrogen chloride in tetrahydrofuran.



(4) (5) (6) Scheme 2

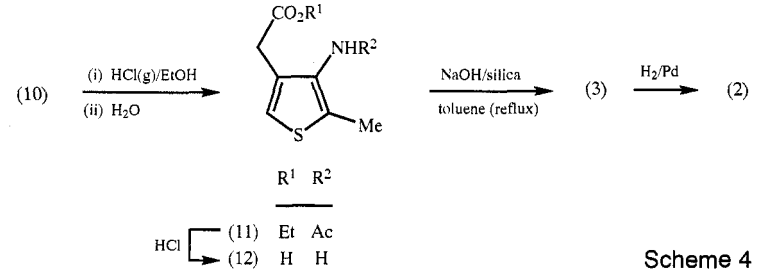
[5] Mosandl, A., Hener, U., and Fenske, H.-D., *Liebigs Ann. Chem.*, 1989, **9**, 859.
[6] Williams, D. H., and Fleming, I., 'Spectroscopic Methods in Organic Chemistry' 3rd Edn, p. 56 (McGraw-Hill: London 1980).
[7] Wolff, L., *Justus Liebigs Ann. Chem.*, 1902, **322**, 351.
[8] Cheney, L. C., and Piening, J. R., *J. Am. Chem. Soc.*, 1945, **67**, 729.

The amine (6) was protected as the acetamide by treatment with acetyl chloride in pyridine in the presence of catalytic 4-dimethylaminopyridine, and the ester acetamide (7) was cleanly reduced with lithium aluminium hydride in tetrahydrofuran at 0° to give 4-acetylamino-5-methylthiophen-3-methanol (8) in an overall yield of 91%. Treating the alcohol (8) with methanesulfonyl chloride in dimethylformamide and collidine did not lead to the expected methanesulfonate but instead gave a low yield of the chloride (9). This is an example of a counterattack reaction,[9] where the initially formed mesylate is reattacked by chloride ion to give the final product. We therefore treated the alcohol (8) with methanesulfonyl chloride as before, but with added lithium chloride to ensure an excess of chloride ion, and obtained 3-acetylamino-4-chloromethyl-2-methylthiophen (9) in 63% yield. Nucleophilic substitution of the chloride (9) by sodium cyanide in dimethylformamide gave 4-acetylamino-5-methylthiophen-3-acetonitrile (10) in 68% yield (Scheme 3), with 14% recovery of the unreacted chloromethyl precursor (9).



Scheme 3

The nitrile (10) was resistant to aqueous hydrolysis under a range of conditions; however, treatment with hydrogen chloride in ethanol under Pinner conditions[10] formed the imidate *in situ* which upon aqueous workup gave ethyl 4-acetylamino-5-methylthiophen-3-acetate (11) in 75% yield. Concomitant hydrolysis of the ester and the acetamide with dilute hydrochloric acid at 70° gave 4-amino-5-methylthiophen-3-acetic acid (12) in 91% yield (Scheme 4).

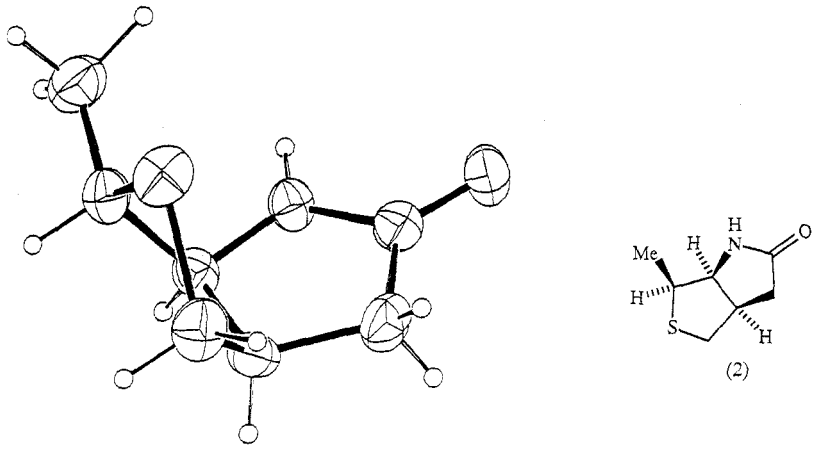


Scheme 4

Various attempts to cyclize the ester (11) by heating with or without solvent were unsuccessful. Ring closure of the amino acid (12) by treatment with either thionyl chloride or under dehydrating conditions (e.g. acetic anhydride, *p*-toluenesulfonic acid in benzene with a Dean–Stark trap) met with little success, while peptide

[9] Hwu, J. R., and Gilbert, B. A., *Tetrahedron*, 1989, **45**, 1233.
[10] Pinner, A., and Klein, F., *Ber. Dtsch. Chem. Ges.*, 1877, **10**, 1889.

coupling reagents such as dicyclohexylcarbodiimide[11] (and modifications thereof)[12] and *N*-cyclohexyl-*N'*-[2-(4-methylmorpholinio)ethyl]carbodiimide tosylate[13] gave the cyclized product in about 15% yield. Dehydration and cyclization on silica in toluene heated at reflux[14] gave 6-methyl-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one (3) in 45% yield. Hydrogenation (6800 kPa) of the thiophen ring in (3) in the presence of an equivalent of 5% palladium on carbon in propan-2-ol gave (3aα,6β,6aα)-6-methyltetrahydro-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one (2) in 20% yield. The structure and all-*cis* configuration of the crippled biotin (2) were confirmed by X-ray crystallography (Fig. 2).



(2)

Fig. 2. Molecular structure of (2) (for structural data see Mackay, M. F., Henderson, S. A., and Savage, G. P., *Acta Crystallogr., Sect. C*, 1995, in press).

Enzyme Binding and In Vivo *Activity*

Compounds (2) and (3) were tested as inhibitors of wheat acetyl–CoA carboxylase and the transcarboxylase component of *Escherichia coli* acetyl–CoA carboxylase, and for herbicidal activity by determining the growth inhibition of *Arabidopsis thaliana*. The acetyl–CoA carboxylase activity was determined at 3 mM MgATP (20 times K_m), 15 mM $NaHCO_3$ (15 times K_m) and 0·05 mM acetyl–CoA (3 times K_m); conditions that are most sensitive to inhibitors of the carboxy-transfer site. Under these conditions the I_{50} values for (2) and (3) were 12·2±1·8 and 1·3±0·2 mM, respectively. These compounds were relatively weak inhibitors of this enzyme compared to the substrates (MgATP and acetyl–CoA) and to biotinol (I_{50} 0·2 mM). They were especially weak when compared to the two commercial classes of graminicides which have I_{50} values that range from 0·00003 to 0·002 mM under these conditions.[15*] Compound

* See also Rendina and Felts (1988).[4]

[11] Sheehan, J. C., and Hess, G. P., *J. Am. Chem. Soc.*, 1955, **77**, 1067.
[12] Frérot, E., Coste, J., Pantaloni, A., Dufour, M.-N., and Jouin, P., *Tetrahedron*, 1991, **47**, 259.
[13] Sheehan, J. C., and Hlauka, J. J., *J. Org. Chem.*, 1956, **21**, 439.
[14] Bladé-Font, A., *Tetrahedron Lett.*, 1980, **21**, 2443.
[15] Rendina, A. R., Felts, J. M., Beaudoin, J. D., Craig-Kennard, A. C., Look, L. L., Paraskos, S. L., and Hagenah, J. A., *Arch. Biochem. Biophys.*, 1988, **265**, 219.

(2) was a slightly better inhibitor of this enzyme than biotin (I_{50} 14·3 mM) or imidazolidone (I_{50} 26 mM), while compound (3) was a better inhibitor than biotin, imidazolidone and parabanic acid (I_{50} 1·74 mM), and showed comparable inhibition to glycoluril (I_{50} 0·88 mM). Biotin analogues must compete with covalently bound biotin, an effect which may explain the relatively weak inhibtion by (2), (3), unbound biotin and some of its analogues.

The *E. coli* transcarboxylase catalyses the reversible formation of acetyl–CoA from malonyl–CoA in the presence of protein-bound biotin (biotin carrier protein), unbound biotin (at low pH) or unbound biotin analogues (at neutral pH) in which the carboxy group is altered (biotin methyl ester, biocytin or biotinol). At 1 mM, compounds (2) and (3) did not inhibit the transcarboxylase when assayed at K_m levels of malonyl–CoA and biotinol. In contrast, the herbicidal graminicides, quizalofop, diclofop and trifop yielded I_{50} values of 0·27, 0·73 and 2·6 mM, respectively. The lack of inhibition at 1 mM suggested that the crippled analogues of biotin were not bound more tightly than biotin or its active analogues which themselves were poorly recognized by transcarboxylase (K_m values *c.* 5 mM for biotin methyl ester, biocytin and biotinol).

The herbicidal activity of compounds (2) and (3) was also very weak. Compound (2) had no effect on *Arabidopsis* growth at any of the rates, while compound (3) showed no effect on growth at 1 and 10 ppm, and only inhibited growth by 43% at 100 ppm. This was extremely weak activity compared to standard herbicides which were active in the 0·01–1 ppm range in this test, and no further testing was conducted. Furthermore, the weak herbicidal activity exhibited by (3) may not be due to inhibiton of acetyl–CoA carboxylase because the *in vitro* inhibition was so weak. Lastly, the weak inhibiton of the two enzymes by (2) and (3) diminished the utility of these compounds as probes for the biotin-binding sites of these enzymes.

Experimental

Melting points were taken in open capillary tubes and are uncorrected. ^{1}H and ^{13}C n.m.r. spectra were recorded at 200 and 50 MHz, respectively, $CDCl_3$ being used as solvent unless otherwise stated. Chemical ionization (c.i.) mass spectra were obtained on a JEOL JMS-DX303 instrument, methane being used as the reagent gas, and electron impact (e.i.) mass spectra were obtained at 70 eV by using a Hewlett–Packard 5890 gas chromatagraph fitted with a 5970 series mass-selective detector. Combustion analyses were performed by the Australian Microanalytical Service, Melbourne. Commercially available reagents were used without further purification and solvents were distilled prior to use.

Acetyl–CoA Carboxylase and Transcarboxylase Inhibition Assays

The procedures used for isolation of acetyl–CoA carboxylase from wheat seedlings[15] and for partial purification (through the phosphocellulose column step) of the transcarboxylase component of acetyl–CoA carboxylase from *E. coli*[16] were based on published methods. Acetyl–CoA carboxylase was stored at −80° in 50 mM K-HEPES, pH 8·5, containing 1 mM dithiothreitol and 20% (v/v) glycerol. Transcarboxylase was stored at −80° in 50 mM K-Pi, pH 7·0, containing 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol and 20% (v/v) glycerol.

Acetyl–CoA carboxylase activity was measured spectrophotometrically in a coupled assay for ADP formation at 25° by determining the decrease of absorbance at 340 nm caused by the oxidation of NADH in the presence of excess pyruvate kinase (Boehringer Mannheim)

[16] Guchhait, R. B., Polakis, S. E., and Lane, M. D., *Methods Enzymol.*, 1974, **35**, 32.

and lactate dehydrogenase (Sigma) as described previously.[17] Transcarboxylase activity was measured spectrophotometrically in the direction of acetyl–CoA formation in a coupled assay at 25° by determining the increase in absorbance at 340 nm caused by reduction of NAD^+ in the presence of excess malate dehydrogenase (Boehringer Mannheim) and citrate synthase (Boehringer Mannheim) by a modification of the literature procedure.[16] Reaction mixtures (0·5 ml) contained 10% ethanol (v/v), 100 mM K-HEPES, pH 7·5, 2 mM L-malate, 0·1 mM NAD^+, 3 U malate dehydrogenase, 3 U citrate synthase, 5 mM biotinol (the biotin analogue in which the carboxy group has been reduced to an alcohol), 0·01 mM malonyl–CoA (to initiate the reaction) and 0·002 U *E. coli* transcarboxylase. Background activity (usually less than 1%) determined in the absence of malonyl–CoA was subtracted from data for each reaction. Potential inhibitors were dissolved in 100% ethanol. Varying portions of this solution were included in both enzyme assay mixtures and the total ethanol concentration was adjusted to 10% (v/v), a level that did not inhibit either enzyme. The concentrations (of compounds) which caused 50% inhibiton (I_{50} values) were determined by linear regression analysis of Dixon plots of the data.[15]*

Bioassay Method

Herbicidal activity (growth inhibition) was determined by means of an *Arabidopsis* bioassay. *Arabidopsis thaliana* seeds were planted on sterile 0·8% agar containing nutrient solution in 12-well titre plates as described in the literature.[17] Test compounds were dissolved in Me_2SO and added to the agar before it hardened (final concentrations were 0, 1, 10 and 100 ppm). The plates with seeds were incubated at 26° under continuous fluorescent lighting and the treatments were visually rated after 3 weeks.

Methyl 5-Methyl-4-oxotetrahydrothiophen-3-carboxylate (5)

Sodium (2·6 g, 0·113 mol) was dissolved in anhydrous methanol (100 ml). A solution of ethyl 2-[2-(methoxycarbonyl)ethylthio]propanoate[5] (15·2 g, 69 mmol) in methanol (50 ml) was added dropwise and the mixture allowed to warm to room temperature. After 2 h the solution was concentrated under reduced pressure to give a yellow slurry which was dissolved in water (150 ml); citric acid solution (*c.* 10%) was added until a pH of approximately 4. The mixture was extracted with dichloromethane (4×100 ml), the combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give an equilibrating mixture of *cis*, *trans* and enol isomers of *methyl 5-methyl-4-oxotetrahydrothiophen-3-carboxylate* (5) as a clear viscous liquid (8·4 g, 70%) (Found: $MH^{+\bullet}$, 175·0429. $C_7H_{11}O_3S$ requires $MH^{+\bullet}$, 175·0422). ^1H n.m.r. δ 1·36, t, J 6·1 Hz; 1·44, d, J 6·9 Hz; 3·0–3·7, m; 3·71, s; 4·16, m; 10·91, s. ^{13}C n.m.r. δ 15·19, 17·45, 21·19, 26·55, 26·92, 29·80, 45·66, 45·89, 46·60, 51·56, 52·75, 54·78, 97·98, 126·90, 168·17, 168·42, 169·69, 175·14, 206·40, 207·07. ν_{max} (KBr) 1740, 1670, 1630, 1455, 1350, 1310, 1270, 1240, 1200, 1145 cm^{-1}. Mass spectrum (e.i.) m/z 174 (23%), 88 (76), 87 (44), 59 (100).

Methyl 4-(Hydroxyimino)-5-methyltetrahydrothiophen-3-carboxylate

Methyl 5-methyl-4-oxotetrahydrothiophen-3-carboxylate (5) (22·3 g, 0·128 mol) was added to a solution of hydroxylamine hydrochloride (10·1 g, 0·145 mol) in ethanol (100 ml) and pyridine (9·7 ml, 0·185 mol), and the mixture stirred for 1 h, then concentrated under reduced pressure. The residue was poured into water (500 ml) and chloroform (100 ml). The organic layer was separated and the aqueous layer extracted with chloroform (2×100 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give *methyl 4-(hydroxyimino)-5-methyltetrahydrothiophen-3-carboxylate* as a clear orange liquid (22·6 g, 93%) (Found: $M^{+\bullet}$, 189·0477. $C_7H_{11}NO_3S$ requires $M^{+\bullet}$, 189·0460). ν_{max} (KBr) 3400br, 1730, 1438, 1370, 1325, 1190, 1160, 1040, 945 cm^{-1}. Mass spectrum (e.i.) m/z 189 (35%), 140 (54), 130 (100), 114 (40), 112 (51), 85 (42), 59 (85), 45 (52).

* See also Rendina and Felts (1988).[4]

[17] Somerville, C. R., and Ogren, W. L., in 'Methods in Chloroplast Molecular Biology' (Eds M. Edelman, R. B. Hallick and N.-H. Chua) pp. 129–138 (Elsevier Biomedical: Amsterdam 1982).

Methyl 4-Amino-5-methylthiophen-3-carboxylate (6) (Method A*)*

Methyl 4-(hydroxyimino)-5-methyltetrahydrothiophen-3-carboxylate (22·6 g, 0·120 mol) was dissolved in tetrahydrofuran (100 ml) and hydrogen chloride gas, which had been dried by bubbling through concentrated sulfuric acid, was gently passed through the solution for 30 min. The mixture was stirred overnight under a nitrogen atmosphere and the resulting precipitate collected by filtration. The salt was treated with saturated sodium hydrogen carbonate solution until the evolution of carbon dioxide ceased and then extracted with chloroform (3×100 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give the crude product. Further product was obtained by treating the original filtrate with saturated sodium hydrogen carbonate solution, and extracting as before. The combined crude product was recrystallized from chloroform/hexane to yield methyl 4-amino-5-methylthiophen-3-carboxylate (6)[18] as pale yellow crystals (8·8 g, 43%), m.p. 60° (Found: C, 49·4; H, 5·3%; $M^{+\bullet}$, 171·0347. $C_7H_9NO_2S$ requires C, 49·1; H, 5·3%; $M^{+\bullet}$, 171·0354). ^{1}H n.m.r. δ 2·17, s, 3H; 3·82, s, 3H; 4·53, br, 2H; 7·71, s, 1H. ^{13}C n.m.r. δ 11·15, 51·32, 110·71, 121·22, 128·20, 142·06, 164·02. ν_{max} (KBr) 1705, 1610, 1415, 1280, 1210, 1080, 740 cm^{-1}. Mass spectrum (e.i.) *m/z* 171 (94%), 139 (100), 111 (92).

Methyl 4-Amino-5-methylthiophen-3-carboxylate (6) (Method B*)*

Methyl 5-methyl-4-oxotetrahydrothiophen-3-carboxylate (5) (6·67 g, 38 mmol) was dissolved in anhydrous acetonitrile (20 ml) which was deoxygenated by passing a stream of dry nitrogen through the solution for 20 min. Hydroxylamine hydrochloride (3·20 g, 46 mmol) was added and the mixture heated at reflux under nitrogen for 3 h. On cooling, the precipitate which formed was collected by filtration, treated with saturated sodium hydrogen carbonate solution until the evolution of carbon dioxide ceased, and the mixture extracted with chloroform (3×100 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was recrystallized from chloroform/hexane to yield methyl 4-amino-5-methylthiophen-3-carboxylate (3·12 g, 48%) which was identical in all respects to that formed in method *A*.

Methyl 4-Acetylamino-5-methylthiophen-3-carboxylate (7)

Methyl 4-amino-5-methylthiophen-3-carboxylate (6) (4·93 g, 29 mmol) was dissolved in pyridine (10 ml). Acetyl chloride (2·5 ml, 35 mmol) was added dropwise to the solution. A catalytic amount of 4-dimethylaminopyridine was added and the solution stirred for 1 h. The solution was poured into water (100 ml) and the mixture was extracted with dichloromethane (3×50 ml). The combined organic extracts were washed with 10% citric acid solution (2×100 ml), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica with dichloromethane/ethyl acetate (1:1) as eluent to give *methyl 4-acetylamino-5-methylthiophen-3-carboxylate* (7) as a cream crystalline solid (5·62 g, 92%), m.p. 79° (Found: C, 51·0; H, 5·2; N, 6·3%; $M^{+\bullet}$, 213·0456. $C_9H_{11}NO_3S$ requires C, 50·7; H, 5·2; N, 6·6%; $M^{+\bullet}$, 213·0460). ^{1}H n.m.r. δ 2·06, s, 3H; 2·20, s, 3H; 3·70, s, 3H; 7·69, s, 1H. 8·37, s, 1H. ^{13}C n.m.r. δ 14·45, 23·53, 51·63, 125·32, 129·29, 131·08, 131·33, 163·49, 168·05. ν_{max} (KBr) 3260, 1722, 1655, 1255, 1188 cm^{-1}. Mass spectrum (e.i.) *m/z* 213 (27%), 171 (77), 170 (36), 139 (100), 111 (43).

4-Acetylamino-5-methylthiophen-3-methanol (8)

Methyl 4-acetylamino-5-methylthiophen-3-carboxylate (7) (3·54 g, 16·6 mmol) in freshly distilled tetrahydrofuran (20 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (1·5 g, 39·5 mmol) in tetrahydrofuran (50 ml) under nitrogen at 0° and the mixture stirred for 1 h. Saturated sodium sulfate solution (3 ml) was added dropwise until the evolution of hydrogen had ceased. The solution was filtered and the filtrate evaporated to give 3-acetylamino-5-methylthiophen-3-methanol as a yellow crystalline solid. The solid from the filtration was heated to reflux in methanol (100 ml) for 1 h. The mixture was filtered and the filtrate concentrated under reduced pressure to give a second crop. The combined crude product was purified by chromatography on silica with methanol as eluent to give

[18] Barker, J. M., and Huddleston, P. R., U.S. Pat. 4847386 (1989). (*Chem. Abstr.*, 1989, **111**, 7216).

4-acetylamino-5-methylthiophen-3-methanol (8) (3·06 g, 99%), m.p. 123–4° (dec.) (Found: C, 51·8; H, 5·8%; $M^{+\bullet}$, 185·0518. $C_8H_{11}NO_2S$ requires C, 51·9; H, 6·0%; $M^{+\bullet}$, 185·0511). ^1H n.m.r. [$(CD_3)_2SO$] δ 2·02, s, 3H; 2·21, s, 3H; 4·27, d, J 5·7 Hz, 2H; 4·96, t, J 5·7 Hz, 1H; 7·03, s, 1H; 9·16, s, 1H. ^{13}C n.m.r. (CD_3OD) δ 12·87, 22·56, 59·26, 118·97, 130·73, 133·96, 139·80, 172·44. ν_{max} (KBr) 3550–3250br, 1655, 1580, 1415 cm^{-1}. Mass spectrum (e.i.) m/z 185 (20%), 167 (70), 125 (100).

3-Acetylamino-4-chloromethyl-2-methylthiophen (9)

Lithium chloride (5·09 g, 0·120 mol) and 4-acetylamino-5-methylthiophen-3-methanol (8) (20·90 g, 0·113 mol) were dissolved in a mixture of collidine (16·4 ml, 0·124 mol) and anhydrous dimethylformamide (60 ml) under nitrogen. The stirred mixture was cooled to 0° and methanesulfonyl chloride (9·8 ml, 0·127 mol) was added dropwise. After stirring for 3 h and allowing to warm to room temperature the mixture was poured into water (700 ml) and extracted with dichloromethane (2×300 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica with dichloromethane/ethyl acetate (1 : 1) as eluent to give *3-acetylamino-4-chloromethyl-2-methylthiophen* as a white solid (14·5 g, 63%), m.p. 131° (dec.) (Found: C, 47·6; H, 5·0%; $M^{+\bullet}$, 203·0181. $C_8H_{10}ClNOS$ requires C, 47·2; H, 5·0%; $M^{+\bullet}$, 203·0172). ^1H n.m.r. δ 2·17, s, 3H; 2·27, s, 3H; 4·43, s, 2H; 7·04, s, 1H; 7·22, br, 1H. ^{13}C n.m.r. [$CDCl_3$/$(CD_3)_2SO$] δ 11·25, 21·05, 38·29, 118·68, 129·56, 130·97, 133·23, 167·55. ν_{max} (KBr) 3250, 1665, 1540s, 1298 cm^{-1}. Mass spectrum (e.i.) m/z 205 (30%), 203 (82), 163 (40), 161 (100), 126 (67), 125 (43).

4-Acetylamino-5-methylthiophen-3-acetonitrile (10)

3-Acetylamino-4-chloromethyl-2-methylthiophen (9) (14·47 g, 71 mmol) and sodium cyanide (3·80 g, 78 mmol) were dissolved in anhydrous dimethylformamide (20 ml) and stirred under nitrogen overnight. The reaction mixture was poured into water (300 ml) and dichloromethane (200 ml). The organic layer was separated and the aqueous layer extracted with dichloromethane (3×200 ml). The combined organic extracts were concentrated under reduced pressure. The crude product was purified by chromatography on silica with dichloromethane/ethyl acetate (1 : 1) as eluent to give, in order of elution, recovered starting material 3-acetylamino-4-chloromethyl-2-methylthiophen (9) (2·08 g, 14%) and *4-acetylamino-5-methylthiophen-3-acetonitrile* (9·37 g, 68%) (Found: C, 55·5; H, 5·0%; $MH^{+\bullet}$, 195·0587. $C_9H_{10}N_2OS$ requires C, 55·7; H, 5·2%; $MH^{+\bullet}$, 195·0592). ^1H n.m.r. δ 1·99, s, 3H; 2·12, s, 3H; 3·38, s, 2H; 6·89, s, 1H; 8·21, s, 1H. ^{13}C n.m.r. δ 12·12, 17·01, 21·97, 117·12, 118·17, 126·63, 129·36, 133·10, 170·00. ν_{max} (KBr) 3270, 1660, 1530, 1378, 1292 cm^{-1}. Mass spectrum (e.i.) m/z 194 (29%), 152 (100), 151 (72), 43 (82).

Ethyl 4-Acetylamino-5-methylthiophen-3-acetate (11)

4-Acetylamino-5-methylthiophen-3-acetonitrile (10) (1·71 g, 8·8 mmol) dissolved in anhydrous ethanol (40 ml) under nitrogen at 0° was stirred while hydrogen chloride gas, which had been dried by bubbling through concentrated sulfuric acid, was gently passed through the solution for 5 min. The solution was allowed to warm to room temperature and stirred overnight under a nitrogen atmosphere. The solvent was removed under reduced pressure and the residue was poured into water (20 ml) and extracted with chloroform (3×50 ml). The aqueous layer was allowed to stand for 2 h, then extracted with chloroform (3×50 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica with chloroform/ethyl acetate (1 : 1) as eluent to give *ethyl 4-acetylamino-5-methylthiophen-3-acetate* (11) as a yellow solid (1·86 g, 75%), m.p. 90·5° (Found: C, 54·4; H, 6·3%; $MH^{+\bullet}$, 242·0865. $C_{11}H_{15}NO_3S$ requires C, 54·7; H, 6·3%; $MH^{+\bullet}$, 242·0851). ^1H n.m.r. δ 1·19, t, J 7·1 Hz, 3H; 2·05, s, 3H; 2·19, s, 3H; 3·40, s, 2H; 4·07, q, J 7·1 Hz, 2H; 6·79, s, 1H; 7·94, br, 1H. ^{13}C n.m.r. δ 13·03, 13·72, 22·67, 34·23, 60·73, 118·38, 129·89, 130·39, 132·82, 168·53, 171·38. ν_{max} (KBr) 3284, 1736, 1650, 1418, 1192 cm^{-1}. Mass spectrum (e.i.) m/z 241 (19%), 154 (31), 153 (100), 126 (32), 125 (45), 43 (40).

4-Amino-5-methylthiophen-3-acetic Acid Hydrochloride (12)

Ethyl 4-acetylamino-5-methylthiophen-3-acetate (11) (1·60 g, 6·6 mmol) dissolved in 2 M hydrochloric acid (50 ml) and ethanol (10 ml) was heated at 70° for 18 h. The solvent was removed under reduced pressure to give *4-amino-5-methylthiophen-3-acetic acid hydrochloride* (12) as yellow crystals (1·25 g, 91%) [Found: $MH^{+\bullet}$ (free amine), 172·0428. $C_7H_{10}NO_2S$ requires $MH^{+\bullet}$ (free amine), 172·0432]. ^{1}H n.m.r. (D_2O) δ 2·31, s, 3H; 3·64, s, 2H; 4·76, s, H_2O residue; 7·00, s, 1H. ^{13}C n.m.r. (D_2O) δ 15·84, 37·72, 126·71, 126·89, 132·12, 137·74, 179·09. Mass spectrum (c.i.) (free amine) *m/z* 174 (6%), 173 (12), 172 (100), 171 (32), 154 (81), 126 (67).

*6-Methyl-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one (3)*

4-Amino-5-methylthiophen-3-acetic acid hydrochloride (12) (0·904 g, 4·4 mmol) was neutralized with 1·25 M sodium hydroxide solution (3·5 ml) and the solvent removed under reduced pressure. The residue was dissolved in methanol (40 ml) and toluene (120 ml). Silica (Merck silica gel 60, 10 g) was added and the mixture heated to reflux under nitrogen for 5 h. After cooling the mixture was filtered and the silica washed with chloroform (200 ml). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica with chloroform/ethyl acetate (1 : 1) as eluent to give *6-methyl-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one* (3) as a pale yellow solid (0·35 g, 45%), m.p. 228–9° (dec.) (Found: C, 55·1; H, 4·4%; $M^{+\bullet}$, 153·0236. C_7H_7NOS requires C, 54·9; H, 4·6%; $M^{+\bullet}$, 153·0248). ^{1}H n.m.r. [$CDCl_3$/$(CD_3)_2SO$] δ 2·06, s, 3H; 3·05, s, 2H; 6·45, s, 1H; 9·85, br, 1H. ^{13}C n.m.r. [$CDCl_3$/$(CD_3)_2SO$] δ 10·01, 33·22, 106·11, 112·80, 127·72, 137·66, 177·36. ν_{max} (KBr) 3120br, 3025br, 2920w, 2792w, 1716s, 1664s, 1458w, 1380m, 1310s, 1202s, 1100m, 778w, 730m, 662m, 590m, 466w cm^{-1}. Mass spectrum (e.i.) *m/z* 155 (6%), 154 (10), 153 (100), 152 (12), 126 (9), 125 (81), 124 (36), 98 (10), 97 (23), 81 (6), 80 (14), 71 (5), 70 (5), 69 (8), 63 (8), 59 (47), 58 (11), 54 (6), 53 (8), 52 (11), 51 (5), 45 (18).

*(3aα,6β,6aα)-6-Methyltetrahydro-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one (2)*

A suspension of 5% palladium on carbon (0·845 g, 4·0 mmol) in propan-2-ol (20 ml) was stirred in a 500-ml Parr stainless steel bomb charged with hydrogen (6800 kPa) for 1 h. The pressure was released and 6-methyl-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one (3) (60 mg, 3·9 mmol) and concentrated sulfuric acid (0·1 ml) were added. The bomb was recharged with hydrogen (6800 kPa) and heated at 50° for 4 h. On cooling the mixture was neutralized with saturated sodium hydrogen carbonate solution, filtered, and the catalyst was washed with propan-2-ol (20 ml). The filtrate was concentrated under reduced pressure to give the crude product which was purified by chromatography on silica with chloroform/ethyl acetate (1 : 1) as eluent to give *(3aα,6β,6aα)-6-methyltetrahydro-1*H*-thieno[3,4-*b*]pyrrol-2(3*H*)-one* (12 mg, 20%) (Found: $M^{+\bullet}$, 157·0570. $C_7H_{11}NOS$ requires $M^{+\bullet}$, 157·0561). ^{1}H n.m.r. δ 1·33, s, 3H; 4·14, dd, *J* 6, 6 Hz, 1H; 7·12, s, 1H. ^{13}C n.m.r. δ 14·05, 37·72, 39·48, 41·67, 49·05, 64·89, 178·79. ν_{max} (KBr) 3572m, 3500s, 3432s, 3176w, 3092w, 2976w, 2928w, 2890w, 1688s, 1642m, 1620m, 1450w, 1424w, 1386m, 1326m, 1266m, 1226w, 1056w, 958w, 824m, 782w, 716w, 626m cm^{-1}. Mass spectrum (e.i.) *m/z* 159 (5%), 158 (9), 157 (91), 99 (5), 98 (9), 97 (58), 96 (65), 89 (5), 84 (9), 83 (16), 82 (7), 80 (5), 75 (35), 74 (61), 73 (7), 71 (6), 70 (8), 69 (100), 68 (35), 67 (7), 65 (5), 61 (5), 60 (9), 59 (27), 58 (8), 57 (19), 56 (19), 55 (16), 54 (47), 53 (7), 52 (6), 47 (9), 46 (15), 45 (36), 44 (6), 43 (31), 42 (21), 41 (81).

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