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(S)-Aspartate Semi-Aldehyde: Synthetic and Structural Studies

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Abstract: We report a novel synthesis of *(S)*-aspartate semi-aldehyde, *(S)*-ASA, **1**, a key intermediate in the biosynthesis of lysine, based on hydrolysis of an enol ether precursor, *(S)*-2-amino-4-methoxybut-3-enoic acid, **10**. *(S)*-ASA is conveniently and quantitatively liberated from this stable intermediate and can be used in biological studies directly, since methanol is the only side-product. Enzyme inhibition studies and chemical precedent imply a cyclic lactol structure might be significant; heteronuclear multibond coupling (HMBC) measurements, however, are consistent with the hydrate being the predominant species in aqueous solution.
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INTRODUCTION

The enzymes of lysine biosynthesis in plants and micro-organisms have been the subject of continued interest in the literature.[1] Several classes of herbicides and antibiotics have been found to inhibit amino acid biosynthesis,[2] but none has yet been found to interfere with the lysine biosynthetic pathway. Inhibition of these enzymes therefore represents an important target for herbicide and bactericide research.

Particular attention has focussed on the branch point enzyme dihydrodipicolinate synthase (DHDPS), which represents a point of feedback regulation in this pathway.[3] DHDPS is presumed to catalyse the condensation of pyruvate and *(S)*-aspartate semi-aldehyde (*(S)*-ASA, **1**) to form dihydrodipicolinate **2**,[4] an unstable heterocyclic precursor of lysine. The reaction is generally represented as illustrated in Figure 1. Investigations have been carried out on the interaction of DHDPS with pyruvate,[5] but relatively little is known about *(S)*-ASA and the reaction product.

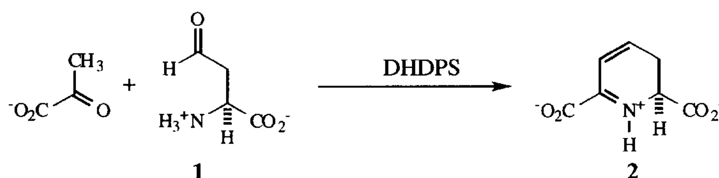


Figure 1

We set out to develop a synthesis of (*S*)-ASA suitable for our enzymological studies, and derive detailed information about biologically significant structural features of (*S*)-ASA, using the pure synthetic samples. The ultimate aim of this work is to examine the mechanistic implications of the condensation reaction with pyruvate and thereby develop (*S*)-ASA analogues as a new class of inhibitor for DHDPS.

RESULTS AND DISCUSSION

A Novel Synthesis of (*S*)-Aspartate Semi-Aldehyde

At the outset of this work the only available literature syntheses of (*S*)-ASA were based on the method of Black and Wright,[6] who employed ozonolysis of (*S*)-allyl glycine in aqueous hydrochloric acid. Characterisation of the product was by enzymatic assay with homoserine dehydrogenase, and chemical characteristion was not attempted. We repeated the literature preparation in both HCl/H_2O and DCl/D_2O in the hope of characterising the product. Enzymatic analysis using a coupled assay of DHDPS and the next enzyme in the pathway, dihydrodipicolinate reductase (DHDPR),[7] suggested that the yield of the reaction in our hands was 88%. The samples of ASA prepared by this method were clearly impure and not suitable for either accurate kinetic measurements or detailed chemical characterisation. One noteworthy feature was the lack of spectral features corresponding to an aldehyde functionality in the ^1H NMR spectrum .

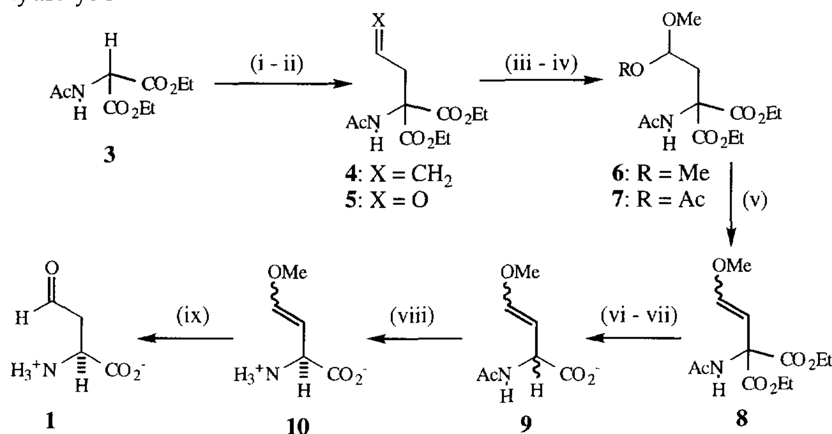
We sought an alternative synthesis of (*S*)-ASA, more appropriate for detailed chemical characterisation, especially for investigations of the structure of (*S*)-ASA in aqueous solution. In addition, we required the immediate precursor to be stable, for convenient storage, and simply and cleanly converted to (*S*)-ASA, without liberation of side-products which could interfere with our enzyme studies. Our approach was via hydrolysis of (*S*)-2-amino-4-methoxybut-3-enoic acid, **10**, a stable, white crystalline solid. Enol ether **10** is not a substrate for DHDPS, nor does it inhibit either DHDPS or DHDPR, although it is a known herbicide.[8] Since enol ethers are hydrolysed under mild acidic conditions,[9] we anticipated that (*S*)-ASA could be liberated in a form suitable for both chemical and biochemical analysis. The side-product, methanol, does not interfere with our assay system.

(*S*)-2-Amino-4-methoxybut-3-enoic acid, **10**, was synthesised using a modification of the method of Keith *et al.*[8] The later synthesis due to Alks and Sufrin[10] was not employed since, in our hands, it proved less efficient than the original synthesis, and the separation of the *E* and *Z* isomers of **10**, included in this synthesis, was not necessary for(*S*)-ASA generation.

Diethyl acetamidomalonate, **3**, was deprotonated using sodium ethoxide in ethanol and allylated;[11] these conditions proved higher yielding than a more recent synthesis employing DMF as a solvent,[12] in particular minimising the formation of a side product: ethyl 2-acetamidopent-4-enoate. The latter arose via dealkylation and decarboxylation of the product, **4**, due to the high nucleophilicity of bromide in the dipolar aprotic solvent. Diethyl 2-acetamido-2-(2-oxoethyl)propanedioate, **5**, was prepared by oxidative cleavage of diethyl 2-acetamido-2-(2-propenyl)propanedioate, **4**, via a Lemieux reaction rather than ozonolysis.[13] The yield of the aldehyde, **5**, was 93% on 20g scale after careful optimisation. If left in the freezer overnight, white

crystals formed from the oil which, after washing with ice-cold diethyl ether, had a higher melting point than those obtained after recrystallisations from ethanol.

After conversion of aldehyde **5** to acetal **6**, the more labile hemiacetal ester, **7** was formed in near quantitative yield on treatment with acetic anhydride and a cationic exchange resin. In our hands the reactions took significantly longer than reported in the literature. Conversion to (*S*)-2-amino-4-methoxybut-3-enoic acid **10**, proceeded uneventfully via pyrolysis, decarboxylation, hydrolysis and pig kidney acylase-mediated resolution.[8] The enol ether was generated as a mixture of geometrical isomers which were not separated since both are converted into (*S*)-ASA by the subsequent hydrolysis.



(i) NaOEt/EtOH; CH_2=$CHCH_2Br$; (ii) $OsO_4/NaIO_4$ (**4** -> **5**); (iii) $HC(OMe)_3$/MeOH/NH_4Cl;
(iv) Ac_2O/Dowex (**6** ->**7**); (v) Δ; (vi) NaOMe/MeOH; (vii) LiOH (aq); (viii) pig kidney acylase; (ix) H_3O^+

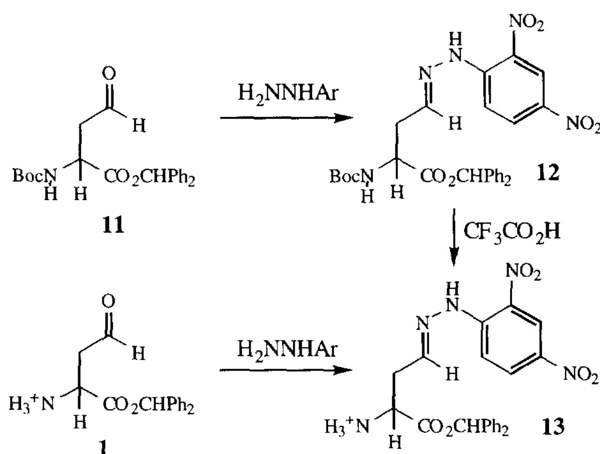
Liberation of (*S*)-ASA from enol ether **10** was achieved using aqueous solutions of hydrochloric acid, 2,4,6-trinitrobenzenesulfonic acid or trifluoroacetic acid. ^1H NMR analysis of hydrolyses carried out in D_2O indicated that all these reactions proceeded quantitatively. This fact was confirmed using our coupled DHDPS/DHDPR assay system. The corresponding salts of (*S*)-ASA could be isolated as solids but, unfortunately, crystallisation of these has not yet proved successful, despite numerous attempts.

Hydrolysis of (*S*)-2-amino-4-methoxybut-3-enoic acid, **10**, represents a novel method of generating (*S*)-ASA. Furthermore, the synthesis is readily achieved from a stable intermediate, and, since the liberation reaction proceeds quantitatively and has only methanol as a side-product, it can be used for the direct generation of (*S*)-ASA for enzymological studies; this contrasts with the requirement for removal of deprotection side-products inherent in the, otherwise admirable, recent synthesis by Tudor *et al.*[14] Samples of (*S*)-ASA prepared by both methods were found to have identical spectroscopic properties.

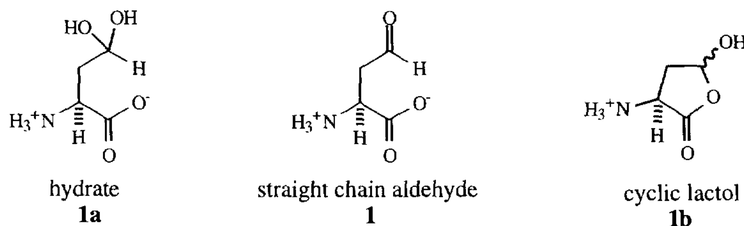
The Solution Structure of (*S*)-Aspartate Semi-Aldehyde

Knowledge of the structure of (*S*)-ASA recognised by DHDPS is essential to enzyme inhibitor design. In the absence of spectroscopic data on (*S*)-ASA, research in this area has traditionally made the tacit assumption that DHDPS recognises the aldehyde, **1**, as its substrate.[4] However, in the

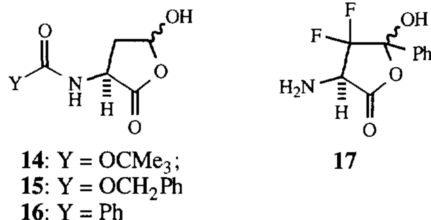
original synthesis of (*S*)-ASA by Black and Wright[6] it is noted: "the usual aldehyde derivatives did not form readily with this substance", a fact which is consistent with all the observed spectral data and casts doubt on this assumption. We re-evaluated the derivatisation chemistry of (*S*)-ASA. Standard tests with semicarbizide, benzaldehyde, dimedone, hydroxylamine and 2,4-dinitrophenylhydrazine[15] all proved negative. Extensive efforts allowed the isolation of trace amounts of a 2,4-dinitrophenylhydrazone; the yield was estimated at less than 0.2%. This material had identical spectroscopic properties to an authentic sample prepared via an alternative route using diphenylmethyl (*N*-*t*-butoxycarbonyl)-2-amino-4-oxobutanoate, **11**. The latter was prepared by diprotection and Lemieux oxidation of (*S*)-allyl glycine; the corresponding 2,4-D.N.P. derivative, **12** was deprotected with trifluoroacetic acid to give the required hydrazone, **13**. It is clear that the free aldehyde is not a chemically significant species in aqueous solutions of (*S*)-ASA.



Recent work by Tudor *et al.*[14] concluded from spectral data that the molecule exists predominantly as a hydrate, **1a**. The form of (*S*)-ASA recognised by DHDPS is not known. In addition to the above structures (aldehyde and hydrate), a further possibility exists which is consistent with the observed chemistry: a cyclic lactol form, **1b**.

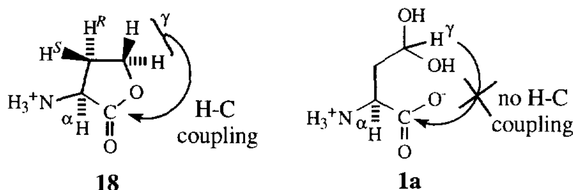


Data on this class of compounds are sparse; however, a study on succinic semi-aldehyde derivatives by Salmon-Legagneur[16] concluded that the acyclic form is a very minor component of an equilibrium which favours a five-membered cyclic lactol. Further, several compounds of this general type have been reported in the literature in lactol form *e.g.* **14**,[17] **15**,[18] **16**[19] and **17**.[20]



We sought information on two issues: do cyclic or acyclic forms of (*S*)-ASA predominate in aqueous solution; and does DHDPS recognise a cyclic or acyclic form of (*S*)-ASA. To address the first issue, structural information about (*S*)-ASA was gleaned using a variety of techniques. ^{1}H NMR, ^{13}C NMR, IR and mass spectra were all consistent with those reported by Tudor *et al.*[14] These measurements do not preclude the existence of a cyclic lactol in aqueous solution. To address this problem, spectroscopic measurements were compared to those for homoserine lactone, **18**, an analogue of **1b**. IR spectra of aqueous solutions of **1** or **18** were not well resolved. The absence of a sizeable IR absorbance expected for a γ-lactone (homoserine lactone in a KBr disc absorbs strongly at 1774 cm^{-1} and in aqueous solution an absorbance at 1784 cm^{-1} can be detected) is consistent with the hydrate, rather than a lactol being the predominant form of (*S*)-ASA in solution. However these measurements were not definitive. Proton NMR afforded more detailed information. Increasing the pH of the solution to 7 and above suggested that traces of the free aldehyde are present under physiological conditions; in particular a resonance at δ_H 9.61 was consistent with a small concentration of free aldehyde.

Heteronuclear multibond coupling (HMBC) NMR experiments were undertaken as a means of distinguishing cyclic and acyclic species. Clear coupling was observed between the carbonyl carbon and the α proton, the pro-*S* β proton (stereochemistry assigned on the basis of nOe measurements), and both the γ protons of homoserine lactone. Analogous coupling was observed between the carbonyl carbon and the α and β protons of (*S*)-ASA; but there was no evidence of coupling between the carbonyl carbon and the γ-proton. This is consistent with the predominant solution structure of (*S*)-ASA being the hydrate **1a**, as suggested by Tudor *et al.*,[14]



Although it appears that only small amounts, if any, of the cyclic lactol **1b** are present we were still interested to evaluate whether such a cyclic structure could be biologically significant. We therefore examined the effect of homoserine lactone and homoserine, analogues of the cyclic and acyclic forms of (*S*)-ASA respectively, on the activity of DHDPS. Concentrations of homoserine of up to 100 mM had no effect on the activity of DHDPS when added to the reaction. By contrast, homoserine lactone was found to inhibit the rate of DHDPS-catalysed reactions at concentrations of

less than 5 mM; with 50 mM homoserine lactone approximately half the enzyme activity was lost The exact inhibition parameters are not yet known since homoserine lactone hydrolyses to homoserine under the standard assay conditions. The rate of hydrolysis was found to correlate with loss of inhibition. These results indicate that DHDPS does interact with γ-lactones derived from amino acids and raises the possibility that a cyclic lactol structure for (*S*)-ASA *might* be biologically significant.

It appears likely that (*S*)-ASA exists primarily as the hydrate, **1a**, in solution. There is evidence for the presence of small amounts of the free aldehyde, **1**, but none for the cyclic lactol **1b**. The search for evidence of a cyclic lactol has lead to the identification of a new type of inhibitor of DHDPS: homoserine lactone. The observed inhibition of DHDPS could be due to homoserine lactone acting as an analogue of **1b**; in which case DHDPS appears to be recognising a minor form of (*S*)-ASA. Alternatively homoserine lactone may be acting as an allosteric inhibitor of the pathway, in keeping with its hypothesised role as a starvation signal in metabolic pathways.[21] Further enzymological studies are underway to distinguish these possibilities.

ACKNOWLEDGEMENTS

Generous funding by Shell Research made the initial stages of this work possible. We gratefully acknowledge Professor Sir John Cornforth F.R.S. for most useful discussions and, in particular, for the key insight that acid-catalysed hydrolysis of (*S*)-2-amino-4-methoxybut-3-enoic acid would provide an effective synthesis of (*S*)-aspartate semi-aldehyde. We also thank Stephen Thomas for assistance with some aspects of the chemistry, Dr John Blunt for carrying out the HMBC experiments, and Dr Peter Steel for helpful comments on the manuscript.

EXPERIMENTAL

All solvents were distilled before use. Flash column chromatography was performed using Merck Kieselgel 60, 230-400 mesh. Thin layer chromatography was performed on Merck Kieselgel DC-Alufolien 60 F_{254} aluminium-backed plates with visualisation by U.V. light. Melting points were determined on a Gallenkamp hot stage apparatus and are uncorrected. Infra-red spectra were recorded on a Perkin Elmer 1600 and 1750 FTIR spectrophotometers. Only selected absorbances are listed. Microanalysis was performed in the Dyson Perrins Laboratory by Mrs V. Lamburn. ^{1}H NMR spectra were recorded on either a Varian Unity, a Bruker AM250 or a Bruker AM500 instrument at 300 MHz, 250 MHz and 500 MHz, respectively, and are referenced to residual solvent peak. ^{13}C NMR spectra were recorded on a Varian Unity or a Bruker AM500 instrument at 75 and 125 MHz, respectively, and are referenced to residual solvent peak. Mass spectra were recorded on V.G. Micromass ZAB1F, V.G. Micromass 20-250, V.G. Masslab 20-250 and Kratos MS80RFA instruments. Only the major peaks are reported, with intensities quoted as percentages of the base peak. U.V. spectra were recorded on Perkin-Elmer 555 and Cary 3 U.V.-visible spectrophotometers. Optical rotations were measured on a Perkin-Elmer 241 polarimeter.

Inhibition studies and biochemical analysis of (*S*)-ASA used a modification of the method of Shedlarski and Gilvarg.[22] The *dap A* and *dap B* genes (encoding DHDPS and DHDPR respectively) were cloned into pBluescript and both enzymes were expressed in *E. coli*. Full details of the over-expression and preparation of DHDPS and DHDPR, together with the modified assay procedure, will be published elsewhere.

Ozonolysis of allyl glycine[6]

Allyl glycine (1.00 g, 8.7 mmol) was dissolved in 1M HCl (25 ml) and chilled to 0°C. Ozone was bubbled through a frit into the chilled mixture for 3 hours, until the solution no longer absorbed ozone as judged by peroxide indicator paper. Oxygen was then bubbled through the solution for 45 minutes to dispel any remaining ozone. The solution was only stable if kept in acidic conditions at <4°C. Purification by Dowex chromatography did not separate the product from formaldehyde (as judged by 2,4-D.N.P. derivatisation). FAB mass spectra showed many peaks: the most abundant were m/z 118 (MH^+) and 100 (MH^+-H_2O). U.V. and I.R. spectroscopy were not fruitful. Removal of the solvent *in vacuo* appeared to result in polymerisation.

Diethyl acetamido-2-(2-oxoethyl)propanedioate, **5**

Diethyl 2-acetamido-2-(2-propenyl)propanedioate[11], **4**, (20.62 g, 80 mmol) was dissolved in a solution of 3:1 dioxane:water (240 ml). Catalytic OsO_4 (<10 mg) was added and the mixture stirred at room temperature until a dark brown/black colouration indicated formation of the osmate ester (30 minutes). $NaIO_4$ (51.36 g, 240 mmol) was added carefully over 45 minutes. The resulting solution was stirred at room temperature until no starting material remained by t.l.c. (diethyl ether). The solution was filtered to remove the white solid which was washed with both ethyl acetate and water. The remaining solution and the washings were partitioned between ethyl acetate and water (5 x 200 ml). The combined organic layers were dried over magnesium sulfate and filtered through a silica plug to remove any traces of OsO_4. Removal of the solvent *in vacuo* left a pale brown oil (19.20 g, 93%). When left overnight at -20°C white crystals formed in the oil; these were separated by filtration, followed by repeated rinsing with ice-cold diethyl ether. The crystals of the title compound, **5**, (m.p. 74°C) were dried under vacuum for several hours after which time the m.p. had increased to 89-90°C (*cf* lit. 80-81°C[13]). R_f(diethyl ether)=0.25; ν_{max}($CHCl_3$) 3405w, 3105w, 2995w, 1745s, 1680s, 1505m, 1480w, 1310w cm^{-1}; δ_H (200 MHz., $CDCl_3$) 1.25 (6H, t, J=7.0 Hz., 2 x $OCH_2C\underline{H}_3$), 2.01 (s, 3H, $NHCOC\underline{H}_3$), 3.66-3.76 (2H, m, $C\underline{H}_2CHO$), 4.25 (4H, q, J=7.0 Hz., 2 x $OC\underline{H}_2$), 7.27 (1H, bs, $N\underline{H}$), 9.67(1H, s, $C\underline{H}O$); m/z [C.I. (NH_3)] 260 (MH^+, 100%).

Diethyl 2-acetamido-2-(2′-acetoxy-2′-methoxyethyl) propanedioate, **7**[8]

Dowex 50*8(H) (8.8g, H^+form, washed sequentially with water, methanol and diethyl ether and dried *in vacuo* over phosphorus pentoxide) was added to a solution of diethyl 2-acetamido-2-(2',2'-dimethoxyethyl)propanedioate, **6**, (12.36 g, 40.4 mmol, prepared from **5** according to a literature procedure[8]) in freshly distilled acetic anhydride (50 ml). The mixture was heated at 65°C. After 1.5 hours (the specified literature period) the reaction had gone to approximately 10% completion as judged by ^1H n.m.r. After heating for a further 18.5 hours, the resin was removed by filtration and the solution evaporated *in vacuo* to give diethyl 2-acetamido-2-(2'-methoxy-2'-acetoxyethyl)propanedioate, **7**, (13.44 g, 99.6%): δ_H (300 MHz., $CDCl_3$) 1.23-1.27, (6H, m, 2 x $C\underline{H}_3$), 2.05 (6H, s, 2 x $C\underline{H}_3CO$), 2.78-2.83 (2H, m, $C\underline{H}_2$), 3.33 (3H, s, $C\underline{H}_3O$), 4.20-4.26 (4H, m, 2 x $OC\underline{H}_2$), 5.72 (1H, t, J=6.0 Hz., $OC\underline{H}O$), 6.83 (1H, bs, $N\underline{H}$).

(S)-*Aspartate semi-aldehyde,* **1**

(S)-2-Amino-4-methoxybut-3-enoic acid **10** (prepared from **7** by pyrolysis, decarboxylative methanolysis, hydrolysis, and pig kidney acylase-mediated resolution according to literature

procedures[8]) was dissolved in 1 M hydrochloric acid. ^{1}H NMR spectra of hydrolyses carried out in D_2O indicated quantitative conversion to *(S)*-ASA, **1**, over 24 hours at 4°C. Enzymatic analysis confirmed that the hydrolysis reaction is essentially quantitative. The hydrolysis could also be mediated by aqueous 2,4,6-trinitrobenzenesulfonic acid or trifluoroacetic acid. In all cases a salt of *(S)*-ASA could be isolated quantitatively as an amorphous solid. The spectroscopic properties of **1** were identical to those of an authentic sample.[14] Selected properties of the trifluoroacetate salt: $[\alpha]_D^{20}$ + 3.2°; ν_{max} (KBr) 3414 2930, 1522, 1678, 1201, 1143 cm^{-1}; δ_H (300 MHz, D_2O) 1.98-2.09 (1H, m, H-3), 2.13-2.21 (1H, m, H-3), 4.01-4.05 (1H, m, H-2), 5.20-5.23 (1H, m, H-4); δ_C (75 MHz, D_2O) 36.2 (C-3), 49.7 (C-2), 87.7 (C-4), 118 (CF_3), 162.9 ($\underline{C}CF_3$), 171.2 (C-1). HMBC: δ_H *ca.* 2.1, coupling to δ_C 49.7, 87.7 & 171.2; δ_H *ca.* 4.0, coupling to δ_C 36.2, 87.7 & 171.2; δ_H *ca.* 5.2, coupling to δ_C 36.2 & 49.7.
Selected spectroscopic properties of homoserine lactone hydrobromide, **18**: ν_{max} (KBr) 3448, 2976, 1774 cm^{-1}; ν_{max} (H_2O) 1784 cm^{-1}; δ_H (300 MHz, D_2O) 2.13-2.28 (1H, m, H-3 (pro-*R*); nOe to δ_H 4.2 & 4.4), 2.51-2.61 (1H, m, H-3(pro-*S*); nOe to δ_H 4.2), 4.16-4.26 (2H, m, H-4), 4.33-4.40 (1H, m, H-2); δ_C (75 MHz, D_2O) 27.9 (t, C-3), 46.9 (d, C-2), 68.7 (t, C-4), 173 (s, C-1). HMBC: δ_H *ca.* 2.2, coupling to δ_C 46.9 & 68.7; δ_H *ca.* 2.6, coupling to δ_C 46.9 & 173; δ_H *ca.* 4.2, coupling to δ_C 27.9 & 173; δ_H *ca.* 4.4, coupling to δ_C 27.9, 46.9 & 173.

N-t-*Butoxycarbonyl allyl glycine*[12]

Allyl glycine (3.98 g, 35 mmol) was dissolved in water/THF (1:1, 80 ml). [2-(*t*-Butoxy-carbonyloxyimino)-2-phenylacetonitrile] (BOC-ON) (9.37 g, 38 mmol) was added followed by freshly distilled triethylamine (5.24 g, 52 mmol) and the resulting mixture stirred for 2 hours at room temperature. The solution was washed with ethyl acetate (2 x 100 ml), the aqueous layer acidified (pH=2) and partitioned with ethyl acetate (3 x 100 ml). These combined organic layers were dried with magnesium sulphate and the solvent removed *in vacuo* to yield a pale yellow oil (5.49 g, 74%): ν_{max}($CHCl_3$), 3441w, 3052s, 2402s, 1713s, 1456m, 1330m, 1245s, 1162m, 818s cm^{-1}; δ_H (200 MHz., $CDCl_3$) 1.45 (9H, s ($C\underline{H}_3)_3C$), 2.35-2.59 (2H, m, $C\underline{H}_2CH{=}CH_2$), 4.34-4.43 (1H, m, $NC\underline{H}CO_2H$), 5.10-5.25 (2H, m, $C\underline{H}_2{=}CH$), 5.62-5.83 (1H, m, $C\underline{H}{=}CH_2$); m/z [C.I.(NH_3)] 233 (MNH_4^+, 9%), 216 (MH^+, 11%). The oil was used without further purification.

*Diphenylmethyl (*N-t*-butoxycarbonyl)-2-aminopent-4-enoate*

To *N-t*-butoxycarbonyl allyl glycine (5.50 g, 24 mmol) dissolved in 50 ml diethyl ether, was added diphenyldiazomethane (5.50 g, 28 mmol) and the mixture stirred at room temperature for 50 minutes. The diethyl ether was evaporated *in vacuo* to yield a yellow solid (10 g). The crude product was absorbed onto silica and purified by column chromatography; eluted with hexane, followed by 19:1 hexane: diethyl ether, then hexane: ethyl acetate: 10:1; 8:1; 4:1 and finally 2:1 . The first compound to elute was excess diphenyldiazomethane; the second was the titled product, (5.60g, 61%): R_f (19:1 hexane:diethyl ether) =0.25; ν_{max} ($CHCl_3$): 3440w, 3000w, 1740s, 1710s, 1495m, 1370m, 1160m cm^{-1}; δ_H (200 MHz., $CDCl_3$) 1.45 (9H, s, $(C\underline{H}_3)_3C$), 2.50-2.65 (2H, m, $C\underline{H}_2CH{=}CH_2$), 4.46-4.59 (1H, m, $NHC\underline{H}CO_2$), 4.97-5.15 (2H, m, $C\underline{H}_2{=}CH$), 5.48-5.65 (1H, m, $C\underline{H}{=}CH_2$), 6.91 (1H, s, $Ph_2C\underline{H}$), 7.1-7.4 (10H, m, $(C_6\underline{H}_5)_2CH$) (*cf* lit.[23]).

*Diphenylmethyl (*N-t-*butoxycarbonyl)-2-amino-4-oxobutanoate , **11***

Diphenylmethyl (*N-t*-butoxycarbonyl)-2-amino-4-pentenoate (4.20 g, 11 mmol) was dissolved in 3:1 water/dioxan (320 ml); catalytic OsO_4 (<1 mg) was added and the mixture stirred at room temperature for 30 minutes; a blue-black colour appeared during this time. $NaIO_4$ (8.80 g, 41 mmol) was added slowly over 30 minutes. The reaction was stirred at room temperature for 6 hours. The reaction mixture was partitioned between ethyl acetate (250 ml) and water (3 x 250 ml), the organic layer was evaporated *in vacuo* to yield 3.8 g of a black oil; this was left under vacuum for 1 hour, then dissolved in $CHCl_3$ and filtered through a silica plug to remove any last traces of OsO_4. The product, **11**, was purified by flash silica chromatography: the oil was charged onto a column equilibrated with hexane and elution effected with a hexane: diethyl ether gradient: 3 column volumes 10:1; 4 volumes 4:1; 4 volumes 1:1; then neat diethyl ether. The titled product **11** eluted last (2.48 g, 59%); t.l.c. R_f (diethyl ether)=0.5. A sample was recrystallised from diethyl ether to yield a white solid: m.pt. 85°C; elemental analysis, expected for $C_{22}H_{24}O_6N$: C 68.9%, H 6.52%, N 3.66%; found: C 69.02%, H 6.83%, N 3.56%. The product was stable for several weeks if kept in diethyl ether solution at -20°C, but was prone to oxidation to the carboxylic acid.

*Diphenylmethyl (*N-t-*butoxycarbonyl)-2-amino-4-oxobutanoate 2,4-dinitrophenylhydrazone, **12***

Diphenylmethyl (*N-t*-butoxycarbonyl)-2-amino-4-oxobutanoate, **11**, (0.25 g, 0.65 mmol) was dissolved in $CHCl_3$ (10 ml) and added to an excess of 2,4-D.N.P. solution[15] which was stirred vigorously overnight at room temperature. No starting material remained after this time, as judged by t.l.c. (diethyl ether). A sticky yellow precipitate formed which was filtered and dried in a desiccator. A drop of benzaldehyde was added to the filtrate to produce a cloudy orange precipitate, confirming that 2,4-D.N.P. had indeed been present in excess. The yellow solid (0.36 g, 0.65 mmol, 100% crude yield) was charged onto a flash silica column equilibrated with 50:50 hexane:CH_2Cl_2 and eluted with 3 column volumes of this solvent followed by a gradually increasing concentration of CH_2Cl_2 to 100%. The titled product **12** eluted last; removal of the solvent *in vacuo* left a bright yellow solid (0.26 g, 72%): m.p. 137-140°C; R_f (CH_2Cl_2) =0.20; ν_{max} ($CHCl_3$) 3600w, 2990m, 1740m, 1750m, 1710s, 1620s, 1590s, 1495m, 1340m, 1160m, 1155m, 1100m, 670m cm^{-1}; δ_H (200 MHz., $CDCl_3$) 1.42 (9H, s ($C\underline{H}_3)_3C$), 3.03-3.05 (2H, m, $C\underline{H}_2CH{=}N$), 4.80-4.83 (1H, m, N$\underline{H}$), 5.38-5.42 (1H, m, C$\underline{H}$NHCO), 6.91 (1H, s, C$\underline{H}Ph_2$), 7.22 (1H, s, N=C$\underline{H}$), 7.51 (1H, d, J=8.0 Hz., Ar$\underline{H}$), 7.32 (10H, br s, $(C_6\underline{H}_5)_2$), 8.10 (1H, dd, J=2.5 Hz., 8.0 Hz., Ar$\underline{H}$), 9.07 (1H, d, J=2.5 Hz., Ar$\underline{H}$), 10.85 (1H, s, N$\underline{H}$).

*Deprotection of diphenylmethyl (*N-t-*butoxycarbonyl)-2-amino-4-oxobutanoate 2,4-dinitrophenylhydrazone **12***

Diphenylmethyl (*N-t*-butoxycarbonyl)-2-amino-4-oxobutanoate 2,4-dinitrophenylhydrazone **12** (0.100 g, 18 mmol) was dissolved in dry CH_2Cl_2 (6 ml) and chilled to 0°C. Methoxybenzene was added (2 drops) followed by trifluoroacetic acid (6 ml) and the mixture stirred on ice for 30 minutes until no starting material remained by t.l.c. (CH_2Cl_2). The resulting amino acid **13** was extracted into water (3 x 10 ml) and the solvent removed *in vacuo*. δ_H (200 MHz., D_2O) 2.95 (2H, m, $C\underline{H}_2C{=}N$), 4.28 (1H, m, $C\underline{H}NH_2COOH$), 7.51-7.64 (2H, m, N=C$\underline{H}$ + Ar$\underline{H}$), 8.08 (1H, dd, J=2.0 Hz., 10 Hz., Ar$\underline{H}$), 8.86 (1H, d, J=2.0 Hz., Ar$\underline{H}$). Attempted recrystallisation of this compound from water and ethanol was not successful.

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