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Canavanine derivatives useful in peptide synthesis

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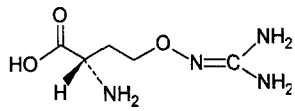
Summary. The objective of this work is to investigate the possibilities for introducing the currently used Nα-, N_G- and C-protective groups into the canavanine molecule and the preparation of canavanines selectively blocked at the guanidino function. These novel compounds will find application in the synthesis of canavanine derivatives expected to be amino acid antimetabolites and of canavanine modified biologically active peptides.

Keywords: Amino acids – Canavanine – Canavanine derivatives-antimetabolites – Alkaline protease – Penicillin amidase

Introduction

Canavanine (**Cav**) **1**, a structural analogue of arginine is the only naturally occurring amino acid containing a guanidinooxy group in its molecule. It has been isolated from more than 500 species of the bean family and is mostly found in its free state, but also as a component of the polypeptide chains of various proteins of plant origin. Its structural resemblance to arginine allows canavanine to compete with it in numerous regulatory and catalytic reactions of the arginine metabolic pathway causing significant disturbances in processes and is thus an effective arginine antimetabolite (Rosenthal, 1975, 1982).



1

Only a few canavanine derivatives have been described so far, most of them salts, a methyl ester (Kitagawa et al., 1932; Gulland et al., 1935), cyclic compounds (Kitagawa et al., 1937; Rickert et al., 1968), an amide, and also an Nα-benzoyl and an acetyl derivatives (Nakatsu, 1959a,b). Obviously, none of the above substances can be directly used for either the preparation of bio-

logically active canavanine derivatives or as peptide chemistry reagents for the synthesis of canavanine containing peptides.

The objective of this work is to investigate the possibilities for introducing the currently used Nα-, N_G- and C-protective groups into the canavanine molecule and the preparation of canavanines selectively blocked at the guanidino function and (or) with an activated carboxyl group. These novel compounds should find application in the synthesis of canavanine derivatives expected to be amino acid antimetabolites, and of canavanine-modified biologically active peptides.

Material and methods

Melting points were determined on a Buchi apparatus and are uncorrected. Elemental analysis and NMR spectra were compatible for all new products synthesized. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. IR spectra were recorded on a Bruker IFS, 113 spectrophotometer with KBr pellets. ^{1}NMR spectra were recorded on Bruker CXP-FT, (200 MHz) instrument using TMS as standard. Reactions were monitored by thin layer chromatography using aluminium-backed Silicagel $60F_{254}$ plates (Merck). Compounds were visualized by U.V., ninhydrin, trisodium pentacyanoammonioferrate (PCAF) and chlorotolidine spray. Solvent systems for TLC were: (a) chloroform:methanol:water, 80:30:5 by vol.; (b) benzene:acetone: acetic acid, 100:50:2 by vol.; (c) chloroform:methanol: acetic acid(32%), 65:45:20 by vol.; (d) 1-butanol:acetic acid:water, 4:1:5 by vol.; (e) ethanol:acetic acid:water, 65:1:34 by vol.

Nα-tert.-Butyloxycarbonyl-canavanine

Boc-Cav-OH (**2**)

1 was dissolved (2.74 g, 10 mmol) in a mixture of 2-PrOH (30 ml), water (10 ml) and TEA (4.2 ml, 30 mmol) with stirring. $(Boc)_20$ (2.9 ml, 13 mmol) was added and stirring was continued until completion of the reaction (about 2 h, TLC monitoring). The organic solvent was evaporated in vacuo, and the cooled aqueous phase was then acidified to pH 2–3 with 1N HCl. The resulting mixture was extracted with n-BuOH (4 × 20 ml). The organic layers were combined, washed with brine, dried over Na_2SO_4 and evaporated in vacuo. The product was precipitated by addition of petrol ether. Recrystallization from EtOH/ether provided 1.95 g (71%); R_f(a) 0.29; $[\alpha]_D^{22}$-19.6 (c 1, DMF); IR (KBr): 3420, 2970, 2940, 1720, 1679 (C=N), 1529, 1359 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.75 ppm (s(br), 4H, guanidino-NH), 5.84 ppm (d, 1H, urethane-NH), 4.00 ppm (m, 1H, α-CH), 3.85 ppm (m, 2H, γ-CH_2), 1.82 ppm (m, 2H, β-CH_2), 1.38 ppm (s, 9H, t-butyl-CH_3).

Nα,N^G-Di-tert.-butyloxycarbonyl-canavanine

Boc-Cav(Boc)-OH (**3**)

1 (1.37 g, 5 mmol) was dissolved in 30 ml of Na_2CO_3 (3.2 g, 30 mmol), and the solution of $(Boc)_2O$ (3.06 g, 14 mmol) in 30 ml dioxane was added with stirring. The stirring was continued until completion of the reaction (3 h; checked by TLC). The organic solvent was evaporated in vacuo, and cooled aqueous phase was then acidified to pH 2–3 with $NaHSO_4$. The resulting mixture was extracted with AcOEt (4 × 20 ml). Organic layers were combined, washed with brine, dried over Na_2SO_4 and evaporated in vacuo. The product was precipitated by addition of petrol ether. Recrystallization from AcOEt/n-

hexane provided 1.70 g (94%); R_f(a) 0.66; $[\alpha]_D^{22}$-25.3 (c 1, DMF); IR (KBr): 3420, 2934, 2840, 1717 (C=N), 1529, 1359 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.10 ppm (d, 1H, urethane-NH), 6.15 ppm (s(br), 3H, guanidino-NH), 3.90 ppm (m, 1H, α-CH), 3.75 ppm (m, 2H, γ-CH_2), 2.03 ppm (m, 2H, β-CH_2), 1.40 ppm (s, 18H, t-butyl-CH_3).

Nα-Benzyloxycarbonyl-canavanine

Z-Cav-OH (**4**)

To a solution of H_2SO_4.H-Cav-OH (2.74 g, 10 mmol) and Na_2CO_3 (1.6 g, 15 mmol) in water (30 ml), Z-OSu (2.50 g, 10 mmol) in 30 ml acetone was added. The mixture was stirred for 3 hours at room temperature. After removing the solvent in vacuo the aqueous layer was acidified with 1N HCl and extracted with AcOEt (3 × 50 ml). The aqueous phase was neutralized to about pH 7 with 5% $NaHCO_3$. Upon concentrating under diminished pressure, crystallization was occurred. After being concentrated to about 10 ml and kept in refrigerator overnight the crystals were collected. The crude product was recrystallized from hot water and dried over P_2O_5. Yield 2.64 g (85%); R_f(a) 0.16; $[\alpha]_D^{22}$-9.6 (c 1, DMF); IR (KBr): 3420, 2970, 2940, 1680 (C=N), 1529, 1359, 748 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.58 ppm (s(br), 4H, guanidino-NH), 7.35 ppm (s, 5H, C_6H_5-Cbz), 6.50 ppm (d, 1H, urethane-NH), 5.12 (s, 2H, CH_2-Cbz), 4.38 ppm (m, 1H, α-CH), 3.82 ppm (m, 2H, γ-CH_2), 2.20 ppm (m, 2H, β-CH_2)

Nα,N_G-Dibenzyloxycarbonyl-canavanine

Z-Cav(Z)-OH (**5**)

1 (2.74 g, 10 mmol) was dissolved in an ice-cold 1N NaOH (10 ml) with stirring. Both stirring and cooling to about O°C was continued while Z-Cl (3,7 ml, 26 mmol) and 2N NaOH (10 ml) were added, in a few portions alternatingly. The pH of the mixture was kept between 9 and 10. After the addition of the reagents, stirring of the suspension was continued for two more hours. The aqueous layer was acidified with 1N HCl and extracted with AcOEt (3 × 50 ml). The AcOEt solution was washed with brine, dried (Na_2SO_4) and evaporated in vacuo. The product was precipitated by addition of PE. Recrystallization from AcOEt/PE. Yield 3.3 g (72%); R_f(a) 0.75, R_f(c) 0.80; $[\alpha]_D^{22}$-31.0 (c 1, DMF); IR (KBr): 3420, 3334, 2970, 2934, 1726 (C=N), 1691, 1502, 1359, 740 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.60 ppm (d, 1H, urethane-NH), 7.35 ppm (s, 10H, 2 × C_6H_5-Cbz), 6.12 ppm (s(br), 3H, guanidino-NH), 5.00 (s, 4H, 2 × CH_2-Cbz), 4.08 ppm (m, 1H, α-CH), 3.78 ppm (m, 2H, γ-CH_2), 1.80–2.04 ppm (m, 2H, β-CH_2).

Nα-9-Fluorenylmethoxycarbonyl-canavanine

Fmoc-Cav-OH (**6**)

1 (2.74 g, 10 mmol) was dissolved in 20 ml water in the presence of 2.78 ml TEA (20 mmol). To this mixture a solution of Fmoc-OSu (3.03 g, 9 mmol) in 20 ml acetonitrile was added in one portion. The pH of the reaction mixture was maintained at pH 8–8.5 by the addition of triethylamine. After stirring for 2 hours, the organic solvent was evaporated in vacuo, and the residue was poured into 20 ml of 1.5N HCl with stirring. The resulting mixture was extracted with (4 × 20 ml) AcOEt/n-BuOH (1:1). After washing with water, saturated NaCl, drying over Na_2SO_4, the organic solvent was evaporated in vacuo. The product was crystallized from EtOH/H_2O and dried over P_2O_5. Yield 3.70 g (93%); R_f(a) 0.51, R_f(b) 0.13; $[\alpha]_D^{22}$-13.8 (c 1, DMF); IR (KBr): 3420, 3319, 3020, 2970, 2940, 1680 (C=N), 1529, 1424, 1359, 740 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.58 ppm (s(br), 4H, guanidino-NH), 7.71 ppm (m, 8H, Arom.-H-Fmoc), 5.80 ppm (d, 1H, CH_2-Fmoc), 5.10–5.20 ppm (d, 2H, urethane-NH, CH-Fmoc), 4.25 ppm (m, 1H, α-CH), 3.80 ppm (m, 2H, γ-CH_2), 2.12 ppm (m, 2H β-CH_2).

Nα,N_G-Di-9-Fluorenylmethoxycarbonyl-canavanine

Fmoc-Cav(Fmoc)-OH (**7**)

1 (1.37 g, 5 mmol) was dissolved in 30 ml of 5% Na_2CO_3, and solution of Fmoc-OSu (3.03 g, 9 mmol) in 50 ml acetone was added with stirring. The stirring was continiued until completion of the reaction (4 hours, checked by TLC). The organic solvent was evaporated in vacuo. The resulting mixture was acidified with conc. HCl. The crystalline product was filtered off and washed with water. The crude product was recrystallized from DCM/PE. Yield 2.1 g (68%); R_f(a) 0.50; R_f(b) 0.32; $[\alpha]_D^{22}$-10.1 (c 1, DMF); IR (KBr): 3420, 3319, 3020, 2970, 2940 1717 (C=N), 1616, 1529, 1424, 1330 740 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.10–7.60 ppm (m, 17H, 2 × Arom.-H-Fmoc, urethane-NH), 6.12 ppm (s(br), 3H, guanidino-NH), 5.10 ppm (d, 1H, CH-Fmoc), 4.38 ppm (m, 1H, α-CH), 4.10 ppm (d, 2H, CH_2-Fmoc), 3.82 ppm (m, 2H, γ-CH_2), 2.20 ppm (m, 2H, β-CH_2).

Nα-Methylsulphonylethoxycarbonyl-canavanine

Msc-Cav-OH (**8**)

1 (0.27 g, 1 mmol) was dissolved in 10 ml acetonitrile/ water (4:1) and 0.28 ml (2 mmol) TEA, then solution of Msc-OSu (0.53 g, 2 mmol) in 5 ml acetonitrile was added with stirring. After stirring for 4 hours the organic solvent was evaporated in vacuo and 10 ml brine was added. The aqueous phase was acidified to pH 4 with conc. HCl and extracted with AcOEt. The aqueous phase was neutralized to about pH 7 with 5% $NaHCO_3$. Upon concentrating under diminished pressure, crystallization was occurred. After being concentrated to about 10 ml and kept in refrigerator overnight the crystals were collected. The crude product was recrystallized from hot water and dried over P_2O_5. Yield 0.23 g (68%); R_f(a) 0.11, R_f(d) 0.32; $[\alpha]_D^{22}$-18.3 (c 1, DMF); IR (KBr): 3420, 3349, 2932, 1697 (C=N), 1735, 1525, 1320, 1150 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.75 ppm (s(br), 4H, guanidino-NH), 7.32 ppm (d, 1H, urethane-NH), 4.00–4.28 ppm (m(t), 3H, α-CH, CH_2-OCO-Msc), 3.79 ppm (m, 2H, γ-CH_2), 3.42 ppm (m, 2H, SO_2-CH_2-Msc), 3.09 ppm (s, 3H, CH_3-SO_2-Msc), 1.81–2.13 ppm (m, 2H, β-CH_2).

Nα-Phenylacetyl-canavanine

PhAc-Cav-OH (**9**)

1 (2.74 g, 10 mmol) was dissolved in 15 ml water and TEA (5 ml). To the solution was dropwise added a solution of PhAc-Cl (1.7 g, 13 mmol) in acetone (15 ml) at 0–5°C, keeping the pH between 7.5–8.5 with TEA. The reaction mixture was stirred for one hour at 0–5°C, and then was concentrated in vacuo to remove the organic solvent. The resulting solution was overlayered with AcOEt (50 ml) and acidified to pH 3 with 18% HCl. The aqueous layer was further extracted with n-BuOH (4 × 20 ml). Organic layers were combined, washed with brine, dried over Na_2SO_4 and evaporated in vacuo. The product was precipitated by addition of petrol ether. Recrystallization from n-BuOH/PE provided 2.32 g (79%); R_f(a) 0.29; $[\alpha]_D^{22}$-8.5 (c 1, DMF); IR (KBr): 3420, 3315, 3033, 2967 2940, 1755, 1687 (C=N), 1610, 1479, 1312, 740 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.30–7.80 ppm (m, 5H, Ar-H-PhAc), 7.53 ppm (s(br), 4H, guanidino-NH), 6.18 ppm (d, 1H, urethane-NH), 4.71 ppm (q, 2H, CO-CH_2-PhAc), 4.08 ppm (m, 1H, α-CH), 3.58 ppm (m, 2H, γ-CH_2), 1.5–2.20 ppm (m, 2H, β-CH_2).

General procedure for deprotection of the PhAc-group

To 5 ml 0.2 M substrate solution, 150 mg of immobilized in polyacrylamide gel Penicillin amidase from Antibiotic-Razgrad, Bulgaria (0.1 mmoles/g support) were added. The reaction mixture was shaken at 25°C and pH 7.8, and maintained with 1N KOH.

Nα-9-Fluorenylmethoxycarbonyl, N^G-4-methoxy-2,3,6-trimethylbenzosulphonyl-canavanine

Fmoc-Cav(Mtr)-OH (**10**)

6 (0.21 g, 0.53 mmol) was dissolved in 6 ml water/acetone (1:1) together with TEA (0.14 ml, 1 mmol) under ice-cooling. To this was added Mtr-Cl (0.13 g, 0.50 mmol) in 3 ml acetone, and the mixture was stirred for about 2 hours. After acidification with 10% citric acid, the solvent was evaporated off, and the material was extracted with AcOEt (3 × 20 ml). The layers was washed with satd. NaCl, dried over Na_2SO_4 and concentrated. The residue was triturated with ether to give a precipitate. Recrystallization from AcOEt/ether provided 0.19 g (60%) yield; R_f(a) 0.57;$[\alpha]_D^{22}$-17.3 (c 1, DMF).

Nα-tert.-Butyloxycarbonyl, N^G-4-methoxy-2,3,6-trimethylbenzosulphonyl-canavanine

Boc-Cav (Mtr)-OH (**11**)

2 (0.83 g, 3 mmol) was dissolved in a mixture of 6 ml water/acetone (1:1) and TEA (0.84 ml, 6 mmol) at room temperature, cooled to 0°C and stirred vigorously. To this solution, Mtr-Cl (1.12 g, 4.5 mmol) dissolved in 2.5 ml acetone was added dropwise at 0°C during a period of 15 minutes. After being stirred for 1 hour at 0°C and then for 2 hours at room temperature, the reaction mixture was acidified with 10% citric acid. The required compound was obtained according the procedure describe above for **10**. Recrystallization from AcOEt/PE provided 0.91 g (62%) yield; R_f(a) 0.53; $[\alpha]_D^{22}$-23.1 (c 1, DMF).

Nα-tert.-Butyloxycarbonyl,N_G-9-fluorenylmethoxycarbonyl-canavanine

Boc-Cav(Fmoc)-OH (**12**)

To a solution of Boc-Cav-OH (0.69 g, 2.5 mmol) in 10 ml 5% Na_2CO_3, Fmoc-OSu (1.01 g, 3 mmol) in 10 ml acetone was added. The required compound was prepared according the procedure describe above for **7** and crystallized from AcOEt/PE. Yield 1.10 g (89%); R_f(a) 0.63; $[\alpha]_D^{22}$-17.0 (c 1, DMF).

Nα-9-Fluorenylmethoxycarbonyl,N^G-tert.-butyloxycarbonyl-canavanine

Fmoc-Cav(Boc)-OH (**13**)

6 (1.19 g, 3 mmol) was dissolved in 10 ml 10% Na_2CO_3, and solution of $(Boc)_2O$ (0.86 g, 4 mmol) in 10 ml dioxane was added with stirring. After the completion of the reaction (3 hours), and the standard work-up, the title compound was obtained in 91% (0.82 g) yield from AcOEt/Pe. R_f(a) 0.53; $[\alpha]_D^{22}$-10.4 (c 1, DMF).

Canavanine methyl ester

2HCl.H-Cav-OMe (**14**)

Dry HCl-gas was passed through a suspension of L-Canavanine in absolute methanol without cooling. The reaction mixture was boiled under reflux for about 6 hours. The clear solution was cooled to room temperature and evaporated to dryness in vacuo. The residue was dissolved in absolute methanol and reevaporated to dryness. Recrystallization from small volume of absolute ethanol, yield 81%. R_f(a) 0.53; R_f(d) 0.17; $[\alpha]_D^{22}$-21.7 (c 1, DMF).

Nα,N_G-Di-tert.-butyloxycarbonyl-canavanine methyl ester

Boc-Cav(Boc)-OMe (**15**)

Method 1: The methyl ester **14** (1.62 g, 5 mmol) was dissolved in 30 ml of 5% Na_2CO_3, and a solution of $(Boc)_2O$ (2.76 ml, 12 mmol) in 20 ml THF was added with stirring. After

completion of the reaction, the organic solvent was evaporated in vacuo. The cooled aqueous phase was extracted with PE (3 × 20 ml), and then was acidified with 5% $NaHSO_4$. The resulting mixture was extracted with AcOEt (4 × 20 ml). The ethyl acetate layers were combined, washed with brine, dried over Na_2SO_4 and evaporated in vacuo. The product was precipitated by addition of PE. Yield 1.7 g (87%); R_f(a) 0.77; $[\alpha]_D^{22}$-28.6 (c 1, DMF); IR (KBr): 3420, 2934, 2840, 1717 (C=N), 1529, 1359 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.10 ppm (d, 1H, urethane-NH), 6.15 ppm (s(br), 3H, guanidino-NH), 3.90 ppm (m, 1H, α-CH), 3.70–3.80 ppm (m(s), 5H, γ-CH_2, OCH_3), 2.03 ppm (m, 2H, β-CH_2), 1.40 ppm (s, 18H, t-butyl-CH_3).

Method 2: Boc-Cav(Boc)-OH (1.36 g, 3.6 mmol) was dissolved in DCM (8 ml). To this solution, maintained were added 0.39 g DMAP (3.2 mmol) and 0.3 ml absolute methanol (3.69 mmol) with stirring. The prepared mixture was cooled to between −5 and 0°C in ice/salt bath, and 0.70 g EDCl (3.66 mmol) was added. The mixture was then stirred for a further 2 hours at 0°C and overnight at RT. The organic phase was evaporated in vacuo, diluted with water and extracted with AcOEt (3 × 20 ml). The AcOEt layers were combined, washed with brine, dried over Na_2SO_4 and evaporated in vacuo. The product was precipitated by addition of PE. Yield 1.2 g (92%); R_f(a) 0.77.

Nα,N_G-Di-tert.-butyloxycarbonyl-canavanine benzyl ester

Boc-Cav(Boc)-OBzl (**16**)

5 (0.83 g, 2.2 mmol) was dissolved in DCM (8 ml). To this solution, maintained were added 0.12 g DMAP (1.1 mmol) and 3.1 ml $PhCH_2OH$ (2.1 mmol) with stirring. The product was obtained according the procedure describe above for **15**. Yield 0.9 g (85%); R_f(a) 0.71;$[\alpha]_D^{22}$-2.6 (c 1, DMF); IR (KBr): 3420, 3315, 2937, 1760, 1718 (C=N), 1610, 1456, 1359, 740 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 7.10–7.28 ppm (d(m), 6H, urethane-NH, benzyl-CH's), 6.15 ppm (s(br), 3H, guanidino-NH), 5.16 (m, 2H, CH_2-benzyl), 3.90 ppm (m, 1H, α-CH), 3.70–3.80 ppm (m(s), 5H, γ-CH_2, OCH_3), 2.03 ppm (m, 2H, β-CH_2), 1.40 ppm (s, 18H, t-butyl-CH_3).

General procedure for deprotection of ester groups

10 mmol of **15** (**16**) were dissolved in a mixture of dioxane (20 ml) and water (70 ml) containing 20 mmol $NaHCO_3$. Alkaline protease from *Bacillus subtilis* strain **DY** (100 mg) was added and the mixture was stirred for 2 (12) hours at 37°C. After removal of the dioxane in vacuo, the aqueous phase was acidified with $NaHSO_4$ to pH 3 and extracted with AcOEt (3 × 70 ml). The combined organic phases were washed with water, dried with Na_2SO_4 and the solvent was evaporated in vacuo. The resulting compound **3** was crystallized from appropriate solvent.

Nα,N_G-Di-tert.-butyloxycarbonyl-canavanine succinimide ester

Boc-Cav(Boc)-OSu (**17**)

A solution of **3** (0.94 g, 2.5 mmol), and HONSu (0.31 g, 2.67 mmol) in THF was cooled in ice-water bath and DCC (0.55 g, 2.67 mmol) was added with stirring. External cooling was removed after the first hour, and the reaction allowed to proceed overnight. The separated N,N-dicyclohexylurea was removed by filtration and the solvent was evaporated in vacuo. The residue was dissolved in AcOEt (30 ml), washed with brine, dried over Na_2SO_4 and evaporated in vacuo. The title compound was obtained as a foam from petrol ether. Yield 0.89 g (76%); R_f(a) 0.86; R_f(b) 0.37; $[\alpha]_D^{22}$-17.0 (c 1, DMF); IR (KBr): 3420, 2970, 2930, 1810, 1735, 1717 (C=N), 1529, 1359 cm^{-1}; ^{1}H-NMR($CDCl_3$) δ: 7.10 ppm (d, 1H, urethane-NH), 6.15 ppm (s(br), 3H, guanidino-NH), 3.90 ppm (m, 1H, α-CH), 3.75 ppm (m, 2H, γ-CH_2), 2.03–2.60 ppm (m 6H, β-CH_2, 2 × CH_2-ONSu), 1.40 ppm (s, 18H, t-butyl-CH_3).

Nα,N_G-Di-9-Fluorenylmethoxycarbonyl-canavanine succinimide ester

Fmoc-Cav(Fmoc)-OSu (**18**)

A solution of **7** (0.62 g, 1 mmol), and HONSu (0.13 g, 1.1 mmol) in DMF was cooled in ice-water bath, and DCC (2.06 g, 1.1 mmol) was added according the procedure describe above for **19**. The crude product was twice recrystallised from isopropanol. Yield 0.51 g (71%); R_f(a) 0.95, R_f(b) 0.77; $[\alpha]_D^{22}$-14.0 (c 1, DMF); IR (KBr): 3420, 3020, 2970, 2940, 1810, 1780, 1735, 1717 (C=N), 1616, 1529, 1424, 1330, 740 cm^{-1}; ^{1}H-NMR($CDCl_3$) δ: 7.10–7.60 ppm (m, 17H, 2 × Arom.-H-Fmoc, urethane-NH), 6.12 ppm (s(br), 3H, guanidino-NH), 5.10 ppm (d, 1H, CH-Fmoc), 4.38 ppm (m, 1H, α-CH), 4.10 ppm (d, 2H, CH_2-Fmoc), 3.82 ppm (m, 2H, γ-CH_2), 2.20–2.60 ppm (m, 6H, β-CH_2, 2 × CH_2-ONSu).

Nα,N_G-Di-tert.-butyloxycarbonyl-canavanine

Boc-Cav(Boc)-NH_2 (**19**)

Method 1: Dry ammonia was led in a gentle stream for about two hours over the stirred solution of **15** (3.90 g, 10 mmol) in absolute methanol. The mixture was allowed to stand at room temperature 48 hours. The crystalline material was collected on a filter, washed with absolute methanol, and dried in vacuo over P_2O_5. Yield 3.2 g (82%); R_f(a) 0.89, R_f(b) 0.27; $[\alpha]_D^{22}$-10.2 (c 1, DMF); IR (KBr): 3420, 3290, 2934, 2840, 1735, 1717, (C=N), 1650, 1529, 1359 cm^{-1}; ^{1}H-NMR($CDCl_3$) δ: 7.10 ppm (d, 1H, urethane-NH), 6.15 ppm (s(br), 3H, guanidino-NH), 5.70 ppm (s, 2H, amide-NH), 3.90 ppm (m, 1H, α-CH), 3.75 ppm (m, 2H, γ-CH_2), 2.03 ppm (m, 2H, β-CH_2), 1.40 ppm (s, 18H, t-butyl-CH_3).

Method 2: TEA (0.7 ml, 5 mmol) was added to a stirred solution of Boc-Cav-OH (1.88 g, 5 mmol) in 15 ml DCM. Then, BOP (1.74 g, 5 mmol) was added, followed after a few minutes by 25% aqueous ammonia (0.4 ml, 5.2 mmol) and TEA (0.84 ml, 6 mmol). The reaction was monitored by TLC. After 1 hour, the mixture was diluted with 50 ml DCM, and subsequently washed with 3N HCl, brine, 10% $NaHCO_3$ and water. The organic solution was dried over Na_2SO_4 and evaporated in vacuo. Crystallization from AcOEt/PE. Yield 1.72 g (92%); R_f(a) 0.89.

Nα,N_G-Di-tert.-butyloxycarbonyl-canavanine hydrazide

Boc-Cav(Boc)-$NHNH_2$ (**20**)

Hydrazine hydrate (1.67 ml, 34 mmol) was added to a solution of **15** (3.90 g, 10 mmol) in methanol and the reaction mixture was kept at room temperature for 3 days. The separated hydrazide was collected by filtration, washed with methanol (20 ml) and ether, and dried over P_2O_5. Yield 3.25 g (80%); R_f(a) 0.58; $[\alpha]_D^{22}$-15.0 (c 1, DMF).

Nα,N_G-Di-9-Fluorenylmethoxycarbonyl-canavanine, tert.-butyloxycarbonyl-hydrazide

Fmoc-Cav(Fmoc)-NHNHBoc (**21**)

7 (0.62 g, 1 mmol) and TBTU (0.35 g, 1.1 mmol) were dissolved in 4 ml DMF. A solution of Boc-$NHNH_2$ (0.16 g, 1.2 mmol) in 2 ml DMF and 0.19 ml DIPEA (1.1 mmol) were added with stirring. After stirring for 4 hours, the DMF was evaporated in vacuo to a syrup, which was dissolved in 50 ml AcOEt, and washed subsequently with 5% citric acid, water, 10% $NaHCO_3$ and water. The organic phase was dried over Na_2SO_4 and evaporated in vacuo to a smaller volume. The product was precipitated by addition of PE. Recrystallization from EtOH/H_2O. Yield 0.70 g (96%); R_f(a) 0.95, R_f(b) 0.65; $[\alpha]_D^{22}$-14.1 (c 1, DMF); IR (KBr): 3420, 3000, 2976, 2940, 1810, 1780, 1735, 1717 (C=N), 1679, 1506, 1245, 740 cm^{-1}.

Results and discussion

During the preparation of N-substituted canavanines our major efforts were directed at obtaining the monosubstituted derivatives, because they can conveniently undergo selective protection at the guanidino-function. Compared to the arginine case this reaction is much more difficult due to the great difference in the basicity of the respective guanidino groups. For instance, it is known that the arginine guanidino group is protonated for all pH values lower than 12.5, whereas in canavanine it loses its proton at pH 7. Thus, in water solutions the zwitter ion form is present and in the solid state an additional proton appears at the Nα-amino group and the guanidino group seems uncharged (Boyar et al., 1982).

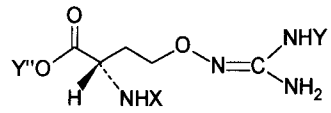
In the compounds with protective groups of the urethane type we were able to determine the reaction conditions for the preparation of the respective mono- and dicanavanine derivatives. Thus, the Nα-tert.-butyloxycarbonyl-canavanine, Boc-Cav-OH **2** was obtained following Pozdnev's method by reaction of canavanine with $(Boc)_2O$ in a water/isopropanol mixture in the presence of TEA at pH 7.5–8.0, or in 5% Na_2CO_3 solution at pH 7.5–8.0, (Pozdnev, 1977) and Nα-9-fluorenylmethoxycarbonyl-canavanine, Fmoc-Cav-OH **6** by treating canavanine with Fmoc-OSu (Carpino et al., 1973) in a water/acetonitrile mixture at pH 7.5–8.5 in the presence of TEA. The respective yields of **2** and **6** were 71% and 93%.

Nα,N_G-di-tert.-butyloxycarbonyl-canavanine, Boc-Cav(Boc)-OH **3** was obtained by interacting canavanine with $(Boc)_2O$ in dioxane medium in the presence of Na_2CO_3 solution, and Nα,N_G-di-9-fluorenylmethoxycarbonyl-canavanine, Fmoc-Cav(Fmoc)-OH **7**, was synthesised by treating canavanine with Fmoc-OSu in acetone in the presence of 5% Na_2CO_3.

Cleavage of the protecting Boc-group was achieved by ethyl acetate saturated with anhydrous HCl (1.5-4N HCl/AcOEt), or TFA/anisole (9:1) in 98% yield. Removal of the Fmoc-protective group was carried out by 50% piperidine in CH_2Cl_2 and yielded 92% for **6** and 96% for **7**.

By using Z-Cl we were unsuccessful in isolating monobenzyloxycarbonyl-canavanine as a main product. In all our experiments significant quantities of mostly di- and also tribenzyloxycarbonyl-canavaine were also present. We were able to obtain only Nα-benzyloxycarbonyl-canavanine Z-Cav-OH **4** by reaction of canavanine with Z-OSu with an yield 85%. Nα-,N_G-dibenzyloxycarbonyl-canavanine, Z-Cav(Z)-OH **5** was obtained by treating canavanine with Z-Cl in a 1N NaOH medium at pH 9 with an yield of 72%.

Reverse reaction of **4** and **5** to canavanine was possible by using 33% HBr/CH_3COOH. Attempted catalytic dissociation of the benzyloxycarbonyl group by 10% Palladium on charcoal in a methanol medium at low hydrogen flow or by the use of formic acid as a hydrogen donor resulted in the breaking of the canavanine molecule into homoserine and guanidine, as evidenced by TLC and the respective standards. These findings confirm Walker's report on the instability of the guanidinooxy group of canavanine subjected to catalytic hydrogenation (Walker, 1955).



where:

Compd.	X	Y	Y″	Compd.	X	Y	Y″
2	Boc	H	H	**12**	Boc	Fmoc	H
3	Boc	Boc	H	**13**	Fmoc	Boc	H
4	Z	H	H	**14**	H	H	Me
5	Z	Z	H	**15**	Boc	Boc	Me
6	Fmoc	H	H	**16**	Boc	Boc	Bzl
7	Fmoc	Fmoc	H	**17**	Boc	Boc	OSu
8	Msc	H	H	**18**	Fmoc	Fmoc	OSu
9	PhAc	H	H	**19**	Boc	Boc	NH_2
10	Fmoc	Mtr	H	**20**	Boc	Boc	$NHNH_2$
11	Boc	Mtr	H	**21**	Fmoc	Fmoc	NHNHBoc

N_a-Methylsulphonylethoxycarbonyl-canavanine, Msc-Cav-OH **8** was obtained in a 68% yield by reacting canavanine with Msc-ONSu in water/acetone (4:1) medium in the presence of TEA (Tesser et al., 1975). Removal of the Msc-protective group was carried out with Tesser's reagent (dioxane/methanol/4N NaOH) (30:9:1) in 96% yield.

Together with base or acid removable protective groups, for the Nα-amino function, enzyme-cleavable protective group are being used lately. One of the most common representatives is the phenylacetyl (**PhAc**) protection. Thus, the Nα-phenylacetyl-canavanine, Nα-PhAc-Cav-OH **9** was synthesised in a 79% yield by treating canavanine with PhAc-Cl in water/acetone medium in the presence of TEA (pH 7.5–8.0). Cleavage of the PhAc-protective group was achived by immobilized *Penicillin amidase* (Fuganti et al., 1986; Waldmann, 1988).

The preparation of the Nα-monosubstituted canavanines **2**, **4**, **6**, **8** and **9** made it possible to achieve selective protection at the guanidino function. The classically used protective groups (viz. nitro, tozyl and the p-methoxybenzylsulphonyl) are applicable only with great difficulty in the case of canavanine due to the specific reaction requirements and equipment (i.e. the presence of anhydrous HF) necessary for the elimination of the protecting groups.

Therefore, we preferred protection by an acid unstable group – 4-methoxy-2,3,6-trimethylbenzo-sulphonyl (**Mtr**) (Fujino et al., 1981; Wakimasu et al., 1982) combined with Fmoc and Boc as Nα-protective groups. Nα-9-Fluorenylmethoxy,N_G-4-methoxy-2,3,6-trimethylbenzosulphonyl-canavanine, Nα-Fmoc,N_G-Mtr-Cav-OH **10** and Nα-tert.-butyloxycarbonyl, N_G-4-methoxy-2,3,6-trimethylbenzosulphonyl canavanine, Nα-Boc,N_G-Mtr-Cav-OH **11** were obtained by treatment of **6** and **9** with Mtr-

Cl respectively in a water/acetone medium (1:1) at 0°C in the presence of TEA. The respective yields of **10** and **11** were 60%, and 62%. Removal of the Mtr protective groups of **10** and **11** was achieved by TFA/anisole (9:1) for 5h at ambient temperature and the respective yields were 96 and 91%.

The reaction of Boc-Cav-OH **4** with Fmoc-OSu in water/dioxane medium in the presence of 10% Na_2CO_3 gave an 89% yield of Nα-tert.-butyloxycarbonyl,N_G-9-fluorenylmethoxycarbonyl-canavanine, Nα-Boc,N_G-Fmoc-Cav-OH **12**. Nα-9-fluorenylmethoxy-carbonyl,N_G-tert.-butyloxycartbonyl-canavanine, Nα-Fmoc,N_G-Boc-Cav-OH **13** was prepared in 91% yield from Fmoc-Cav-OH **6** according to the procedure as for the **4**. The latter is specifically N_G-guanidino protected canavanine derivative, albeit containing the less common protective group.

It is important to determine whether N-protected canavanines undergo substitution at the Nα- or the N_G site. This can be elucidated by comparison of IR- and NMR-spectral data of arginine and canavanine with those of the derivatives. In the IR spectrum of non-substituted canavanine a medium intensity band appears at 1670cm^{-1}, caused by valency vibrations of the (**C=N**) of the guanidino group. Most characteristic are the changes in IR spectra of diacyl canavanine derivatives. The **C=N** stretch was shifted towards the higher frequency regions by 10 to 50cm^{-1} and a new characteristic band appeared at 1726cm^{-1}. No shift has been observed in the case of monoacylated canavanines. It was anticipated that acylation would bring about reduction of the **C=N** stretching frequency. And indeed, the modulation band of the **(C=N)** of Boc-Cav(Boc)-OH is shifted towards the higher frequency regions (1717cm^{-1}), as compared to that of free amino acids. In the proton spectra of canavanine and its derivatives the characteristic signals for the Nα-amino group and the guanidino group are present. The different protecting groups show their characteristic signals.

Among the variety of possible C-protective groups for the carboxyl group we chose the methyl and benzyl groups and synthesised the corresponding esters. The methyl ester of canavanine **14** was obtained with an 81% yield by flowing anhydrous hydrogen chloride through a suspension of the amino acid in absolute methanol. The methyl ester obtained by us had a melting point and $[\alpha]_D$ equal to those reported by Kitagawa et al. (1935). Two methods were used for the preparation of Nα,N_G-di-tert.-butyloxycarbonyl-canavanine methyl ester, Boc-Cav(Boc)-OMe **15**. The required compound was prepared by interacting canavanine methyl ester with $(Boc)_2O$ in THF and aqueous Na_2CO_3 solution, or was synthesised after the method of Dhaon et al. (1982) by reaction of **3** with absolute methanol in the presence of 4-N,N-dimethylamino pyridine (DMAP) and 1-ethyl-3[3-(dimethylamino) propyl]carbodiimide (EDCI) in dichloromethane. The second method affords an yield of 92% (against 87% for the other) and the reaction time is shorter. The benzyl ester **16** of canavanine was also prepared by the Dhaon's method and afforded an yield of 85%. Boc-Cav(Boc)-OMe **15** was saponified in a methanol medium at room temperature for 1 hour evidenced by thimolphtalein, and yielded 98%.

As stated above, catalytic hydrogenolysis is inapplicable to canavanine and its derivatives. Therefore, no attempts were made for removal of the

benzyl ester group by this classical method. As an alternative we preferred the enzyme catalysed saponification of various esters – a method for dissociation of C-protections used in contemporary peptide chemistry (Aleksiev et al., 1981). The saponification of **15** catalyzed by alkaline protease from *Bacillus subtilis* strain **DY** offered the best results. The reaction took 2 hours to afford an 98% yield. Saponification of Boc-Cav(Boc)-OBzl **16** by *Bacillus subtilis* strain **DY** alkaline protease was significantly slower (12h), but nevertheless an yield of 90% was obtained. The enzyme/substrate ratio and all remaining conditions were the same as for Boc-Cav(Boc)-OMe.

We have preferred the activated ester method, and particularly that for N-succinimide esters for the activation of the canavanine carboxyl group. Nα,N$_G$-di-tert.-butyloxycarbonyl-canavanine succinimide ester, Boc-Cav(Boc)-OSu **17** and Nα,N$_G$-di-9-fluorenylmethoxycarbonyl-canavanine succinimide ester, Fmoc-Cav(Fmoc)-OSu **18** were obtained by treatment of **3** and **7** with excess of HONSu and DCC in acetonitrile and DMF respectively, the yields being 76% and 71%.

Using Boc-Cav(Boc)-OMe **15** as a starting substance we have obtained the protected amide Boc-Cav(Boc)-NH_2 **19** and hydrazide Boc-Cav(Boc)-$NHNH_2$ **20** by amonolysis in anhydrous ammonium or hydrazinolysis by hydrazine hydrate, respectively (Pajpanova et al., 1989). The yields were 82% and 80%. Boc-Cav(Boc)-NH_2 **19** may be also obtained by method of Fehrentz et al. (1983) by interaction of Boc-Cav(Boc)-OH with 25% aqueous ammonia in CH_2Cl_2, with BOP (Benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate) as an activator in the presence of TEA. The second method affords an yield of 92% (against 82% for the other) and the reaction time is shorter. Removal of the Nα-, N$_G$-protective groups of **19** and **20** was achieved by 1.5N HCl/AcOEt. The hydrogen chloride salts of canavanine amide and canavanine hydrazide were obtained in 96% and 87% yields, respectively. The interaction of Fmoc-Cav(Fmoc)-OH **7** with Boc-$NHNH_2$ in DMF, with TBTU (O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluroniumtetrafluoroborate) as an activator and N,N-diisopropylethylamine (DIPEA) base afforded 96% yield of Fmoc-Cav(Fmoc)-NHNHBoc **21**.

Canavanine amide and canavanine hydrazide are interesting as possible antimetabolites of arginine. Screening for biological activity of those compounds was the topic of a separate report (Pajpanova et al., 1992). Non-substituted Boc-Cav(Boc)-$NHNH_2$ **19** and the hydrazine substituted canavanine **20** are likely to be important in the synthesis of canavanine-modified proteins.

The canavanine derivatives were obtained in a chromatographic purity (TLC and HPLC) and identified by elemental analysis, Mass-, IR- and ^{1}H-NMR-spectra. In Table 1 the physico-chemical and analytical data of the new compounds are presented.

Conclusion

In conclusion we should mention, that as a result of our investigations a large number of canavanine derivatives have been obtained that may find application both in modern peptide chemistry for the synthesis of canavanine-

Table 1. Analytical data of compounds **2–21**

Comp. no	Formula M.w.	M.p. [°C]	$[\alpha]_D$ DMF c = 1	MS m/z M^+	Analysis(%)			Comp. no	Formula M.w.	M.p [°C]	$[\alpha]_D$ DMF c = 1	MS m/z M^+	Analysis(%)		
					C	Calc./ H	Found N						C	Calc./ H	Found N
2	$C_{10}H_{20}N_4O_5$ (276.294)	88–9	19.6	276	43.37 43.50	7.28 7.59	20.26 20.45	**12**	$C_{25}H_{29}N_4O_7$ (497.527)	135–6	−17.0	497	60.35 60.23	5.87 5.60	11.26 11.15
3	$C_{15}H_{28}N_4O_7$ (376.413)	95–6	−25.3	376	47.86 47.76	7.49 7.70	14.88 14.52	**13**	$C_{25}H^3{}_{29}N_4O_7$ (497.527)	125–6	−10.4	497	60.35 60.38	5.87 5.90	11.26 11.33
4	$C_{13}H_{18}N_4O_5$ (310.316)	210	−9.6	310	50.32 50.02	5.85 5.66	18.06 18.34	**14**	$C_6H_{16}N_4O_3Cl_2$ (263.123)	166–7	−21.7	–	27.39 27.21	6.13 6.13	15.43 15.37
5	$C_{21}H_{24}N_4O_7$ (444.447)	189	−31.0	376	56.75 56.74	5.44 5.64	12.60 12.60	**15**	$C_{16}H_{30}N_4O_7$ (390.448)	128	−28.6	390	49.21 49.13	7.75 7.88	14.35 14.20
6	$C_{20}H_{21}N_4O_5$ (397.406)	81–3	−13.8	397	60.44 60.32	5.32 5.65	14.09 13.93	**16**	$C_{22}H_{34}N_4O_7$ (482.538)	107–8	−2.6	482	54.76 54.86	7.09 7.11	11.61 11.45
7	$C_{35}H_{30}N_4O_7$ (618.637)	174	−10.1	618	67.95 67.75	4.89 5.11	9.06 9.45	**17**	$C_{19}H_{31}N_5O_9$ (473.490)	57–8	−17.0	473	48.19 48.20	6.60 6.85	14.79 14.90
8	$C_9H_{18}N_4O_7S$ (344.372)	156–7	18.3	344	31.39 31.02	5.56 5.67	16.27 16.30	**18**	$C_{39}H_{33}N_5O_9$ (715.718)	118	−14.0	715	65.43 65.30	4.65 4.70	9.78 9.58
9	$C_{13}H_{18}N_4O_4$ (294.310)	115	−8.5	294	53.05 53.21	6.16 6.06	19.03 19.11	**19**	$C_{15}H_{29}N_5O_6$ (375.423)	110	−10.2	375	47.98 48.01	7.78 7.86	18.65 18.60
10	$C_{30}H_{33}N_4O_8S$ (609.673)	−17.3	−17.3	609	59.10 58.83	5.45 5.78	9.19 9.38	**20**	$C_{15}H_{30}N_6O_6$ (390.438)	176	−15.0	390	46.14 46.10	7.74 7.72	21.52 21.35
11	$C_{20}H_{32}N_4O_8S$ (488.555)	170–1	−23.1	488	49.17 48.96	6.60 6.49	11.47 11.41	**21**	$C_{40}H_{40}N_6O_8$ (732.791)	122	−14.1	732	65.56 65.55	5.50 5.23	11.45 11.33

modified peptides and for obtaining canavanine derivatives that may be arginine antimetabolites. The comparative instability of the guanidinooxy-group has caused some difficulties, but they should be easily overcome. Viewed in the light of the advances in current canavanine chemistry and biochemistry, our achievements may considered to be of pioneering character and, therefore we are encouraged to continue studies in this field in the future.

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