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Synthesis of novel aminoisoflavones and their L-alanyl conjugates, for possible use in chromogenic media for the detection of aminopeptidase

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Abstract

Aminoisoflavones are synthetic derivatives of a group of natural products found in plants and dietary components such as soya derived products. Three novel aminoisoflavones have been prepared via nitration of the parent isoflavone followed by reduction using tin (II) chloride dihydrate. These unstable aminoisoflavones were then conjugated with L-alanine, to give stable derivatives. The instability of the parent suggested that these conjugates could be used in chromogenic media to detect L-alanyl aminopeptidase producing Gram-negative bacteria, as cleavage of the amide would result in decomposition and the production of colored compounds. However, biological evaluation with a series of bacteria were conducted, although a simpler o-aminophenol conjugate did give black colonies with Escherichia coli strains on doped agar plates.

1. Introduction

Aminoisoflavones are synthetic derivatives of a group of natural products found in plants and dietary components such as soybeans and soya derived products. These compounds were initially synthesized to determine the effect of the amino substituent on the biological activity of the parent compounds [1]. However, it was observed that the aminoisoflavones were very unstable in the presence of oxygen and rapidly decomposed, going black in the process. The actual products of the decomposition were not identified but were presumably mainly polymeric material. In order to synthesize pure samples of the aminoisoflavones it was thus necessary to work under an inert atmosphere, where all the oxygen had been removed. However, it was also found that the compounds could be stabilized chemically *via* acylation of the amino group [2].

It was intended to examine the possibility of exploiting the instability of the aminoisoflavones to develop a simple and reliable test for L-alanyl aminopeptidase producing Gram-negative bacteria [3]. Attaching L-alanine to the amino group should give stable compounds that are substrates for bacterial endopeptidases [4]. Following enzymatic cleavage, the unstable aminoisoflavone will then be released and rapidly decompose. If the stable substrate is incorporated into agar plates, then the presence of Gram-negative bacteria might result in the appearance of black colonies on the agar which will be easily observed. Literature precedent comes from the work of Manafi *et al.* who used fluorogenic substrates of L-alanyl aminopeptidase, both incorporated into the growth medium and absorbed into paper strips, to test for the presence of Gram-negative bacteria [5]. In Manafi's system illumination was required at 366 nm to observe the fluorescence produced by the best substrate and there were also problems with

background fluorescence form the growth medium. The advantage of our new procedure would be that the black colonies should be easily visible to the naked eye.

2. Material and Methods

2.1. Synthesis

2.1.1. General Information

Melting points were determined in open capillary tubes with an electrothermal apparatus and are uncorrected. NMR spectra were recorded on a Varain Gemini 200 (^{1}H, 200 MHz; ^{13}C 50.31 MHz), Bruker Avance 300 (^{1}H, 300 MHz; ^{13}C 75.46 MHz), or a Bruker Avance 500 spectrometer (^{1}H, 500 MHz; ^{13}C 125.7 MHz). For ^{1}H NMR spectra the residual peaks of $CHCl_3$ (7.26 ppm) and CH_3SOCH_3 (2.59 ppm) were used as internal reference, while for ^{13}C NMR spectra the central peak of $CDCl_3$ (77.0 ppm) and that of DMSO-d_6 (39.95 ppm) were used. Chemical shifts are given in δ and J values in Hz. Peak assignments were performed for the new compounds with the aid of the 2D COSY, GHSQCTOCSY and GHMBC spectra. EI mass spectra were recorded on a VG Autospec and electrospray (CI) mass spectra were recorded using a Micromass LCT instrument.

2.1.2. 7,2',4'-Trimethoxyisoflavone 3

$BF_3.Et_2O$ (5.28 mL, 41.7 mol) was added to a solution of 2,4-dihydroxy-2',4'-dimethoxydeoxybenzoin **1** (2.4 g, 8.33 mmol), synthesized by Friedel-Craft acylation of resorcinol and 2,4-dimethoxyphenylacetic acid as described in the literature,[3] in DMF (20 mL) under nitrogen at ambient temperature. After 15 min stirring a solution of methanesulfunyl chloride (3.26 mL, 33 mmol) in DMF (3 mL) was slowly added. After heating at 75°C for 6 hrs the reaction mixture was cooled down to ambient temperature and poured into ice-cold aqueous sodium acetate (12 g/100 mL). The yellow precipitate was filtered off and recrystallized from aqueous ethanol to give **2** as yellow crystals (2.2 g, 88%), m.p. 293-294 °C; δ_H (300 MHz, DMSO-d_6) 3.69 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 6.55 (dd, *J* = 2.3, 8.4 Hz, 1H, H-5'), 6.62 (d, *J* = 2.3 Hz, 1H, H-3'), 6.85 (d, *J* = 2.2 Hz, 1H, H-8), 6.91 (dd, *J* = 2.2, 8.7 Hz, 1H, H-6), 7.12 (d, *J* = 8.4 Hz, 1H, H-6'), 7.90 (d, *J* = 8.7 Hz, 1H, H-5), 8.12 (s, 1H, H-2), 10.76 (s, 1H, 7-OH). The dry product (2.0 g, 6.7 mmol) and anhydrous potassium carbonate (1.85 g, 13.4 mmol) were then stirred under nitrogen atmosphere and methyl iodide (0.46 mL, 7.38 mmol) was added. After the suspension was stirred for 8 hrs under reflux, the mixture was cooled down to ambient temperature and the solid was removed by filtration. The acetone was removed under reduced pressure, and the yellow residue was recrystallized from EtOH to yield the title compound **3** as a pale-yellow solid (1.95 g, 93%). m.p. 146-148 ºC. δ_H (300 MHz, $CDCl_3$) 3.78 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.54-6.57 (m, 2H, H-3',5'), 6.84 (d, *J* = 2.4 Hz, 1H, H-8), 6.96 (dd, *J* = 8.9, 2.4 Hz, 1H, H-6), 7.25 (d, *J* = 8.9 Hz, 1H, H-6'), 7.88 (s, 1H, H-2), 8.18 (d, *J* = 8.9 Hz, 1H, H-5). Anal. ($C_{18}H_{16}NO_5$) C, H.

2.1.3. 5'-Nitro-7,2',4'-trimethoxyisoflavone 4

Fuming HNO_3 (0.11 mL, 1.76 mmol) was added to a stirred suspension of **3** (0.5 g, 1.6 mmol) in glacial acetic acid (15 mL) under nitrogen at 15 ºC. After 3 hrs stirring the dark-red solution was poured into ice-water (20 mL), the yellow precipitate was filtered, washed with water (50 mL), and dried. The crude product was purified by using column chromatography (silica, CH_2Cl_2: EtOAc, 9:2) to yield of the title compound **4** as a pale yellow solid (0.33 g, 58%). m.p. 239-241 °C (from ethanol); δ_H (300 MHz, DMSO-d_6) 3.90 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3), 6.92 (s, 1H, H-3'), 7.09 (dd, *J* = 2.4, 8.9 Hz, 1H, H-6), 7.18 (d, *J* = 2.4 Hz, 1H, H-8), 7.95 (s, 1H, H-6'), 7.98 (d, *J* = 8.9 Hz, 1H, H-5), 8.36 (s, 1H, H-2); δ_C (75 MHz, DMSO-d_6) 56.1 (C-OCH_3), 56.7 (C-OCH_3), 57.0 (C-OCH_3), 97.33 (C-3'), 100.7(C-8), 113.1 (C-4a), 114.9 (C-6), 117.2 (C-1'), 119.8 (C-3), 126.8 (C-6'), 128.9 (C-5), 131.0 (C-5), 155.1 (C-2), 155.5 (C-4'), 157.4 (C-8a), 162.7 (C-7), 163.8 (C-2'), 174.0 (C-4); *m/z* (EI) 357 (M^+, 100%), 340 (23), 326 (37), 312 (29), 281 (28), 266 (21), 253 (17), 238 (11), 225 (15), 210 (12), 182 (13), 151 (37), 122 (19), 107 (23); HRMS (CI): calcd for ($C_{18}H_{15}NO_7Na$) 380.0746 found 380.0746. Anal. ($C_{18}H_{15}NO_7$) C, H, N.

2.1.4. 3'-Nitrogenistein 7

Fuming HNO_3 (0.26 mL, 6.11 mmol) was added to a stirred suspension of genistein **5** (1.5 g, 5.56 mmol) in glacial acetic acid (15 mL) under nitrogen at 15°C. After 3 hrs stirring at rt the dark-red solution was poured into ice-water (20 mL), the yellow precipitate was filtered, washed with water (50 mL), and dried. Recrystallization from EtOH: acetone (95:5) afforded **7** as yellow crystals (1.2 g, 94%). m.p. 257-258 ºC (from methanol); δ_H (300 MHz, DMSO-d_6) 6.24 (d, J = 2.1 Hz, 1H, H-6), 6.42 (d, J = 2.1 Hz, 1H, H-8), 7.22 (d, J = 8.7 Hz, 1H, H-5'), 7.77 (dd, J = 8.7, 2.3 Hz, 1H, H-6'), 8.17 (d, J = 2.2 Hz, 1H, H-2'), 8.49 (s, 1H, H-2); δ_C (75 MHz, DMSO-d_6) 93.8 (C-8), 99.2 (C-6), 104.3 (C-4a), 118.9 (C-5'), 120.1 (C-3), 121.9 (C-1'), 125.2 (C-2'), 135.5 (C-6'), 136.5 (C-3'), 151.8 (C-4'), 155.0 (C-2), 157.5 (C-8a), 161.9 (C-5), 164.5 (C-7), 179.6 (C-4); *m/z* (EI) 315 (M^+, 100%), 316 (18), 269 (6), 241 (7), 152 (17), 136 (3), 124 (5); HRMS (EI): calcd for ($C_{15}H_9NO_7$) 315.0379, found 315.0383. Anal ($C_{15}H_9NO_7$) C, H, N.

2.1.4. 8-Nitrobiochanin A 8

Fuming HNO_3 (0.147 mL, 3.87 mmol) was added to a stirred suspension of biochanin A **6** (1.0 g, 3.52 mmol) in glacial acetic acid (15 mL) under nitrogen at 15 °C. After 8 hrs stirring at 70 °C the dark-red solution was poured into ice-water (20 mL), the brown precipitate was filtered, washed with water (50 mL), and dried. The crude product was purified by using column chromatography (silica, CH_2Cl_2: EtOAc, 8:2) to yield the title compound as a pale yellow solid (0.62 g, 54%). m.p. 239-241 °C (from ethanol); δ_H (300 MHz, DMSO-d_6) 3.87 (s, 3H, OCH_3), 6.42 (s, 1H, H-6), 7.10 (d, J = 8.8 Hz, 2H, H-3', 5'), 7.52 (d, J = 8.8 Hz, 2H, H-2', 6'), 8.54 (s, 1H, H-2); δ_C (75 MHz, DMSO-d_6) 55.0 (OCH_3), 98.5 (C-6), 103.4 (C-4a), 113.6 (C-3', 5'), 121.7 (C-8, 1'), 122.9 (C-3), 130.1 (C-2', 6'), 149.5 (C-8a), 154.0 (C-2), 157.0 (C-7), 159.3 (C-4'), 162.7 (C-5), 179.5 (C-4); *m/z* (EI) 329 (M^+, 100%), 330 (19), 298 (6), 267 (5), 132 (13), 117 (4); HRMS: calcd for ($C_{16}H_{11}NO_7$) 329.0535, found 329.0534. Anal. ($C_{16}H_{11}NO_7$) C, H, N.

2.1.5. General procedure for reduction of nitroisoflavones to aminoisoflavones

A mixture of the isoflavone and an excess of stannous chloride in 20 mL of absolute ethanol was heated at 70 ºC under Ar. After the starting material has disappeared as indicated by TLC, cold water was added *via* syringe at 20 ºC. The pH was adjusted to 7-8 by the addition of 5% aqueous $NaHCO_3$ and NaH_2PO_4 and the mixture extracted four times with ethyl acetate (100 mL x 4). The combined organic layers were thoroughly washed with brine, treated with charcoal and dried over Na_2SO_4. Removal of the solvent at reduced pressure leaves the amino compound, which gives one spot on TLC. The entire work-up is done under nitrogen owing to the lability of aminoisoflavones.

2.1.6. 3'-Amino-5,7,4'-trihydroxyisoflavone 9

Prepared from **7** (0.5 g, 1.58 mmol) and $SnCl_2.2H_2O$ (2.5 g, 11.1 mmol) to give yellow crystals (0.44 g, 97%). m.p. 234 °C decomp; δ_H (200 MHz, DMSO-d_6) 4.67 (br, 2H, NH_2), 6.23 (d, J = 2.1 Hz, 1H, H-6), 6.39 (d, J = 2.1 Hz, 1H, H-8), 6.58 (dd, J = 8.0, 2.1 Hz, 1H, H-6'), 6.70 (d, J = 8.0 Hz, 1H, H-5'), 6.82 (d, J = 2 Hz, 1H, H-2'), 8.25 (s, 1H, H-2,), 9.28 (br, 1H, OH), 10.98 (br, 1H, OH), 12.56 (s, 1H, OH) ; δ_C (DMSO-d_6) 93.4 (C-8), 98.7 (C-6), 104.2 (C-4a), 113.8 (C-2'), 114.9 (C-5'), 116.9 (C-6'), 121.5 (C-3), 122.8 (C-1'), 136.1 (C-3'), 144.0 (C-4'), 153.4 (C-2), 157.3 (C-8a), 161.8 (C-5), 164.0 (C-7), 180.1 (C-4); *m/z* (EI) 285 (M^+, 100%), 286 (20), 252 (17), 176 (7), 153 (12), 133 (14), 120 (6), 105 (4), 88 (13); HRMS (EI); calcd for ($C_{15}H_{11}NO_5$) 285.0637, found 285.0641.

2.1.7. 8-Amino-5,7-dihydroxy-4'-methoxyisoflavone 10

Prepared from **8** (0.9 g, 2.74 mmol) and $SnCl_2.2H_2O$ (2.31 g, 13.68 mmol) to give **10** as yellow crystals (0.72 g, 95%). m.p. 211 ºC decompose; δ_H (200 MHz, DMSO-d_6)3.85 (s, 3H, OCH_3), 6.37 (s, 1H, H-6), 7.06 (d, J = 8.9 Hz, 2H, H-3', 5'), 7.57 (d, J = 8.9 Hz, 2H, H-2', 6'), 8.43 (s, 1H, H-2), 12.22 (s, 1H, 5-OH0; δ_C DMSO-d_6) 55.1 (C-OCH_3), 98.4 (C-6), 104.08 (C-8), 113.6 (C-3', 5'), 116.0 (C-4a), 121.3 (C-

1'), 123.2 (C-3), 130.0 (C-2', 6'), 143.6 (C-8a), 151.0 (C-7), 151.8 (C-5), 154.0 (C-2), 159.0 (C-4'), 180.5 (C-4); *m/z* (CI) 299 (M+Na^+ 100%); HRMS (CI): calcd for ($C_{16}H_{14}NO_5$) 300.0872, found 300.0874.

2.1.8. 5'-Amino-7-2',4'-trimethoxyisoflavone 5

Prepared from **4** (0.5 g, 1.40 mmol) and $SnCl_2.2H_2O$ (1.66 g, 9.80 mmol) at reflux temperature for 8 hrs in ethanol, yielding **5** as a white solid (0.4 g 97%). Due to its instability the crude product was reacted further without analysis.

2.1.9. S-2-Amino-N-(2-hydroxyphenyl) propionamide. HCl 14

A solution of HOBt.H_2O (0.56 g, 4.12 mmol) and DCC (0.68 g, 3.30 mmol) in dry DMF (5 mL) was added to a solution of 2-aminophenol **11** (0.30 g, 2.75 mmol) in anhydrous DMF (5 mL) at 0 ºC. After 20 min stirring at 0°C, *N-tert*-butoxycarbonyl-L-alanine **12** (0.62 g, 3.3 mmol) was added and the mixture was stirred for overnight while it was gradually allowed to reach rt. The solvent was then removed under reduced pressure. The crude oily residue was purified using column chromatography (silica, petroleum ether: EtOAc 2:1) to furnish compound **13** as a white solid (0.47 g, 61%). m.p. 130-131 ºC; δ_H (500 MHz, $CDCl_3$-d_6) 1.46 (d, *J* = 2.1 Hz, 3H, CH_3), 1.47 (s, 3H, 3 x CH_3), 4.46 (br s, 1H, CH), 5.27 (d, *J* = 6.5 Hz, 1H, HNCO), 6.83 (t, *J* = 7.5 Hz, 1H, H-4), 6.89 (d, *J* = 8.0 Hz, 1H, H-6), 7.08 (t, *J* = 7.75 Hz, 1H, H-5), 7.15 (d, *J* = 6.5 Hz, 1H, H-3), 8.83 (br s, 1H, OH), 8.94 (br s, 1H, HNCO); δ_C (75 MHz, $CDCl_3$) 17.8 (C-CH_3), 28.3 (3 x C, CH_3), 50. 5 (C-CH), 81.18 (C-C(CH_3)$_3$), 119.0 (C-6), 120.41 (C-4), 122.4 (C-3), 125.35 (C-2), 126.9 (C-5), 148.4 (C-1), 156.2 (C-NCOOBoc), 172.60 (C-CO); *m/z* (EI) 280 (M^+, 5%), 224 (33), 207 (16), 162 (26), 147 (29), 135 (74), 120 (52), 109 (100); HRMS (EI): calcd. for ($C_{14}H_{20}N_2O_4$) 280.1423, found, 280.1418. Anal. ($C_{14}H_{20}N_2O_4$) C, H, N.

6N HCl-dioxane (10 mL) was slowly added to a solution to a solution of **13** (1.5 g, 5.36 mmol) in dry dioxane (10 mL) under nitrogen, and stirred at room temperature for 5 hrs. Then dry diethyl ether (10 mL) was added to the suspension, and the precipitate was filtered and washed twice with diethyl ether (20 mL) to give the title compound **14** (0.84 g, 86%). m.p. 238-240 ºC; δ_H (500 MHz, DMSO-d_6) 1.44 (d, *J* = 7.0 Hz, 3H, CH_3), 4.21 (br s, 1H, CH), 6.77 (m, 1H, H-6), 6.96 (m, 2H, H-4,5), 7.80 (d, *J* = 8.5 Hz, 1H, H-3), 8.38 (br s, 3H, NH_3), 9.83 (s, 1H, HNCO), 10.04 (br, 1H, OH); δ_C (125 MHz, DMSO-d_6) 18.7 (C-CH_3), 28.71 (3 x C, CH_3), 49.9 (C-CH), 56.2 (C-OCH_3), 56.4 (C-OCH_3), 56.7 (C-OCH_3), 81.18 (C-C(CH_3)$_3$), 96.2, 100.5, 113.4, 114.8, 118.7, 123.3, 123.7, 128.2, 150.11, 154.0, 155.1, 157.4, 158.5, 164.2, 170.7, 175.9; *m/z* (CI) 181 (MH^+, 100%) HRMS: calcd for ($C_9H_{13}N_2O_2$) 181.0977, found 181.0983. Anal. ($C_9H_{13}N_2O_2Cl$) C, H, N.

2.1.10. S-2-Amino-N-(5,7,4'-trihydroxyisoflavonyl) propionamide

Coupling of 3'-aminogenistein **9** (0.5 g, 1.75 mmol) with **12** according to the procedure described for **11** afforded **15** as light-yellow solid (0.47 g, 75%). m.p. 207-208 ºC; δ_H (500 MHz, acetone-d_6) 1.47 (s, 3H, 3 x CH_3), 1.48 (d, *J* = 2.1 Hz, 3H, CH_3), 2.89 (d, *J* = 7.0 Hz, 1H, NH), 4.46 (br s, 1H, CH), 6.29 (d, *J* = 2.0 Hz, 1H, H-6), 6.42 (d, *J* = 2.0 Hz, 1H, H-8), 6.97 (d, *J* = 8.4 Hz, 1H, H-5'), 7.24 (dd, *J* = 8.4, 2.0 Hz, 1H, H-6'), 8.05 (s, 1H, H-2), 8.17 (d, J = 1.5 Hz, 1H, H-2'), 9.30 (br s, 1H, HNCO), 12.89 (s, 1H, 5-OH); *m/z* (CI) 479 (M+Na^+, 100%) HRMS (CI): calcd for ($C_{23}H_{24}N_2O_8Na$) 479.1430, found 479.1418. Anal. ($C_{23}H_{24}N_2O_8$) C, H, N.

6 N HCl-dioxane (10 mL) was slowly added to a solution to a solution of **13** (1.5 g, 5.36 mmol) in dry dioxane (10 mL) under nitrogen, and stirred at room temperature for 5 hrs. Then dry diethyl ether (10 mL) was added to the suspension, and the precipitate was filtered a1nd washed twice with diethyl ether (20 mL) to give the title compound **18** (0.95 g, 81%). m.p, 261-262 ºC; δ_H (500 MHz, DMSO-d_6) δ1.46 (d, *J* = 2.1 Hz, 3H, CH_3), 4.46 (br s, 1H, CH), 6.29 (d, *J* = 2.0 Hz, 1H, H-6), 6.42 (t, *J* = 2.0 Hz, 1H, H-8), 6.97 (d, *J* = 8.4 Hz, 1H, H-5'), 7.24 (dd, *J* = 8.4, 2.0 Hz, 1H, H-6'), 8.05 (s, 1H, H-2), 8.17 (d, J = 1.5 Hz, 1H, H-3'), 8.38 (br s, 3H, NH_3), 9.8 (s, 1H, OH), 10.3 (s,1H, OH), 11.08 (s, 1H, OH), 12.9 (s, 1H, OH); δ_C (75 MHz, DMSO- d_6) 17.4 (C-CH_3), 48.6 (C-CH), 93.7 (C-6), 99.0 (C-8), 104.3, (C-4a), 115.3 (C-5'), 121.2 (C-3), 122.2 (C-1'), 123.6 (C-2'), (124.8 (C-3'), 125.9 (C-6'), 148.5 (C-4a), 154.1 (C-2), 157.5 (C-8a), 161.9 (C-5), 164.4 (C-7), 168.5 (C-NCO), 172.60 (C-4); *m/z* (CI) 357 (M+H^+, 100%), 214 (36); HRMS (CI): calcd for ($C_{18}H_{17}N_2O_6$) 357.1087, found 357.1097. Anal. ($C_{18}H_{17}N_2O_6Cl$) C, H, N.

2.1.11. 5'-Amino-7,2',4'-trimethxoyisoflavone conjugate (Hydrochloride salt)

Coupling of 5'-amino-7,2',4'-trimethxoyisoflavone **5** (0.4 g, 1.22 mmol) with **12** according to the procedure described for **11,** followed by purification using column chromatography (silica, CH_2Cl_2: EtOAc 8:2), afforded **16** as a light-yellow solid (0.42 g, 69%). m.p. 194-195 ºC; δ_H (300 MHz, $CDCl_3$) 1.43 (d, *J* = 7.1 Hz, 3H, CH_3), 1.47 (s, 3H, 3 x CH_3), 3.80 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.92 (C-2 x CH_3), 4.31 (br s, 1H, CH), 5.07 (br s, 1H, HNCO), 6.57 (s, 1H, H-3'), 6.85 (d, *J* = 2.4 Hz, 1H, H-8), 7.00 (dd, *J* = 8.9, 2.4 Hz, 1H, H-6), 7.81 (s, 1H, H-6'), 8.17 (d, *J* = 8.9 Hz, 1H, H-5), 8.21 (s, 1H, H-2); δ_C (DMSO-*d*$_6$) 18.7 (C-CH_3), 28.71 (3 x C, CH_3), 49.9 (C-CH), 56.2 (C-OCH_3), 56.4 (C-OCH_3), 56.7 (C-OCH_3), 81.18 (C-$\underline{C}(CH_3)_3$), 96.2, 100.5, 113.4, 114.8, 118.7, 123.3, 123.7, 128.2, 150.11, 154.0, 155.1, 157.4, 158.5, 164.2, 170.7, 175.9;); *m/z* (CI) 521 ($M+Na^+$, 100%) HRMS (CI): calcd for ($C_{26}H_{30}N_2O_8Na$) 521.1900, found 521.1902. Anal. ($C_{26}H_{30}N_2O_8$) C, H, N.

6 N HCl-dioxane (10 mL) was slowly added to a solution to a solution of **16** (1.0 g, 5.36 mmol) in dry dioxane (10 mL) under nitrogen, and stirred at room temperature for 5 hrs. Then dry diethyl ether (10 mL) was added to the suspension, and the precipitate was filtered and washed twice with diethyl ether (20 mL) to give the title compound **19** (0.67 g, 84%). m.p. 210-212 ºC**;** δ_H (500 MHz, DMSO-*d*$_6$) 1.44 (d, *J* = 7.0 Hz, 3H, CH_3), 3.77 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.12 (br s, 1H, CH), 6.83 (s, 1H, H-3'), 7.08 (dd, *J* = 9.0, 2.5 Hz, 1H, H-6), 7.16 (d, *J* = 2.5 Hz, 1H, H-8), 7.60 (s, 1H, H-6'), 7.98 (d, *J* = 9.0, 1H, H-5), 8.24 (br s, 3H, NH_3), 8.25 (s, 1H, H-2), 9.71 (s, 1H, HNCO); δ_C (75 MHz, DMSO-*d*$_6$) 17.5 (C-CH_3), 48.5 (C-CH), 56.0 (C-OCH_3), 56.1 (C-2 x OCH_3), 96.8 (C-6), 100.6 (C-8), 112.0 (C-1'), 114.7 (C-3'), 117.4 (C-4a), 118.2 (C-3), 121.4 (C-5'), 126.5 (C-6'), 126.8 (C-5), 152.1 (C-4'), 154.5 (C-2), 155.5 (C-2'), 157.5 (C-8a), 163.7 (C-7), 168 (C-HNCO), 174.3 (C-4); *m/z* (CI) 399 ($M+H^+$, 100%), 328 (84). Anal. ($C_{21}H_{23}N_2O_6Cl$) C, H, N.

2.1.12. 8-Aminobiochanin A conjugate (Hydrochloride salt)

Coupling of 8-aminobiochanin A **10** (0.5 g, 1.67 mmol) with **12** according to the procedure described for **11** followed by purification using column chromatography (silica, CH_2Cl_2: EtOAc 8:2), afforded **17** as a light yellow solid (0.47 g, 60%). m.p. 167-168 ºC; δ_H (300 MHz, $CDCl_3$) 1.49 (s, 3H, 3 x CH_3), 1.52 (d, *J* = 2.1 Hz, 3H, CH_3), 3.43-3.52 (m, 1H, NH), 4.46 (br s, 1H, CH), 5.08 (br s, 1H, NH), 6.45 (s, 1H, H-6), 6.99 (d, *J* = 8.8 Hz, 2H, H-3',5'), 7.44 (d, *J* = 8.8 Hz, 2H, H-2',6'), 7.86 (s, 1H, H-2), 8.81 (d, J = 1.5 Hz, 1H, H-3'), 10.37 (br s, 1H, 7-OH); 12.49 (s, 1H, 5-OH); Anal. ($C_{24}H_{26}N_2O_8$) C, H, N.

6N HCl-dioxane (10 mL) was slowly added to a solution of **17** (1.0 g, 5.36 mmol) in dry dioxane (10 mL) under nitrogen, and stirred at room temperature for 5 hrs. Then dry diethyl ether (10 mL) was added to the suspension, and the precipitate was filtered and washed twice with diethyl ether (20 mL) to give the title compound **20** (0.64 g, 86%). m.p. 219-220 ºC; δ_H (500 MHz, DMSO-*d*$_6$) 1.56 (d, *J* = 7.0 Hz, 3H, CH_3), 3.79 (s, 3H, OCH_3), 4.09 (br s, 1H, CH), 6.48 (d, *J* = 3.0 Hz, 1H, H-6), 7.01 (d, *J* = 8.5 Hz, 2H, H-3,5), 7.49 (d, *J* = 8.5 Hz, 1H, H-2',6'), 8.34 (br s, 3H, NH_3), 8.41 (s, 1H, H-2), 9.76 (s, 1H, HNCO), 11.34 (s, 1H, 7-OH), 12.92 (s,1H, 5-OH); δ_C (75 MHz, DMSO-*d*$_6$) 17.4 (C-CH_3),48.4 (C-CH), 55.2 (C-OCH_3), 98.7 (C-6), 103.0 (C-8), 104.2 (C-4a), 113.7 (C-3',5'), 122.3 (C-1'), 122.5 (C-3), 130.2 (C-2',6'), 153.0 (C-8a), 154.0 (C-2), 159.2 (C-4'), 160.0 (C-7), 160.6 (C-5), 169.5 (C-HNCO), 180.2 (C-4); *m/z* 371 ($M+H^+$, 100%), 353 (26), 336 (11), 328 (10), 300 (22), 196 (6); HRMS: calcd for ($C_{19}H_{19}N_2O_6Cl$) 371.1243, found 371.1242. Anal. ($C_{19}H_{19}N_2O_6Cl$) C, H, N.

3. Results and discussion

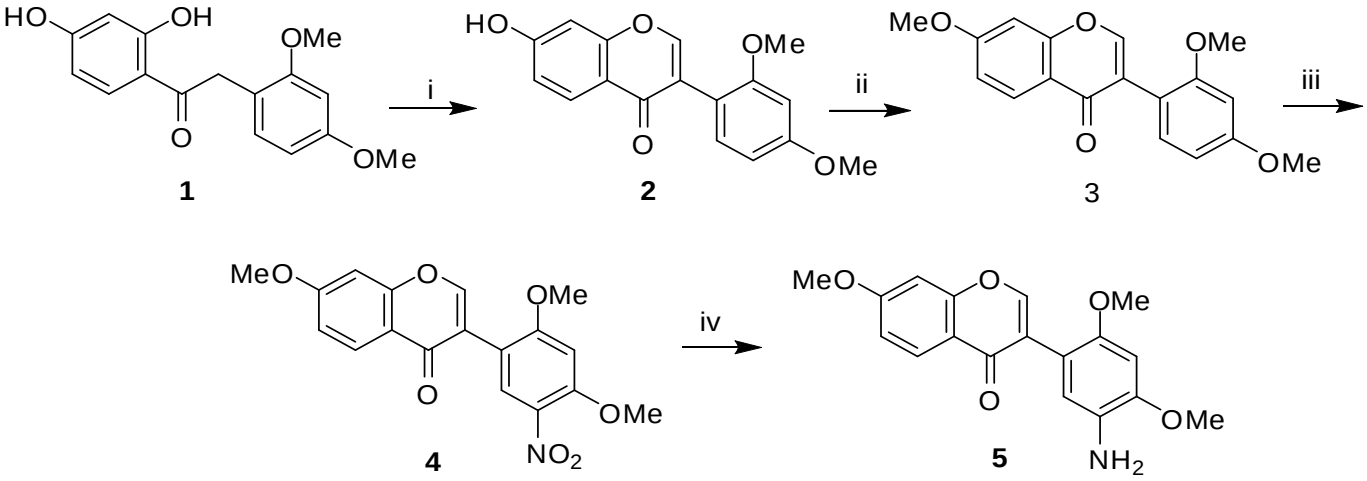
3.1. Synthesis of aminoisoflavones

Three main routes to nitroisoflavones are known in the literature. Nitro-substituted deoxybenzoins [6-8] prepared in moderate yield from the corresponding phenols and nitrophenylacetic acid, may be cyclized to nitroisoflavones but this procedure is restricted to cases where the nitro group is in the non-fused aromatic ring. Another route involves the oxidative rearrangement of nitroflavones in the presence of thallium (III) salts [9] in a polar protic solvent. The disadvantages of this procedure are the high toxicity of the thallium reagent, long reaction times and the poor yields obtained. Electrophilic nitration of

isoflavones should be widely applicable but has only been applied to 6-hydroxyisoflavone [10], 7-hydroxyisoflavone **1e** [10, 11], 6-hydroxy-7-methoxyisoflavone [12] and their methyl ethers, using fuming HNO_3 in the presence of glacial acetic acid or sulfuric acid. As isoflavones are easily accessible from the corresponding phenols and phenylacetic acid [13], their nitration appeared to be the most promising general route to the various nitroisoflavones. The nitroisoflavones can then be reduced using either zinc [7, 14], iron dust [14] or sodium hydrogen sulphite [10] .The reduction has also been achieved by hydrogenation over 10% Pd/C [15] or Raney nickel [12]. However, in our hands these reduction procedures were not entirely satisfactory, particularly for the *ortho*-aminohydroxy isoflavones due to their instability when exposed to air. Complete and selective reduction of the nitroisoflavones **4**, **7** and **8** was successfully achieved employing the mild reducing agent $SnCl_2.2H_2O$ as indicated in Schemes 1 and 2 [16].

3.2. 3'-Amino-7,4',6'-trimethoxyisoflavone

The synthesis of 3'-amino-7,4',6'-trimethoxyisoflavone **5**, where the amino-group required for the coupling with *N*-Boc-L-alanine was present in the electron rich B-ring, is presented in Scheme 1.



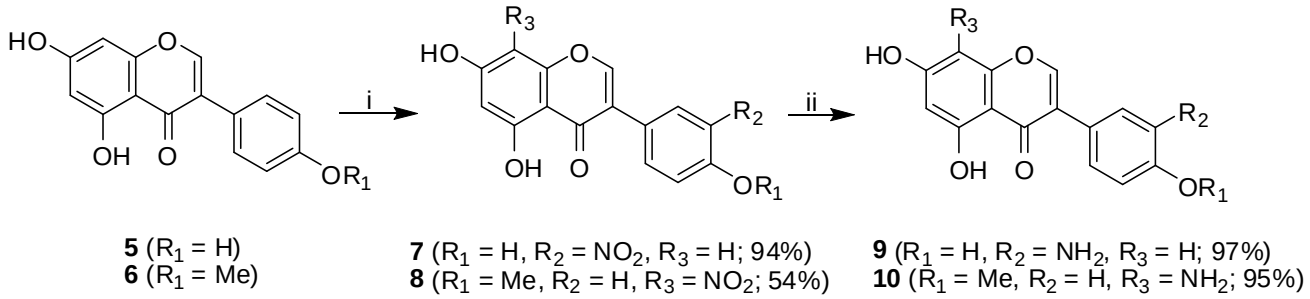
Scheme 1. i) DMF, $BF_3.Et_2O$, $MeSO_2Cl$, 70 ºC (88%); ii) CH_3I, K_2CO_3, acetone, reflux (93%); iii) 1 eq. HNO_3, acetic acid, rt (58%); iv) $SnCl_2.2H_2O$, EtOH, 70-75 ºC (97%, crude).

Formylation and subsequent cyclisation of 1-(2,4-dihydroxyphenyl)-2-(2,4-dimethoxyphenyl) ethanone **1**, which was synthesized according to a literature procedure, was carried out *via* a Vilsmeier-Haack type reaction (DMF, $BF_3.Et_2O$, $MeSO_2Cl$) to give 7-hydroxy-4',6'-dimethoxyisoflavone **2** in 85% yield. Methylation of **2** with iodomethane, followed by treatment of the fully methylated isoflavone **3** with 1 eq. of fuming HNO_3 in acetic acid afforded the 5'-nitro-7,4',6'-trimethoxyisoflavone **4** in a moderate yield.

3.2. Amino derivatives of genistein and biochanin A

Initially good quantities of both genistein and biochanin A were synthesized using literature methods [17]. The nitration of genistein **5** with 1.1 eq. of fuming HNO_3 in acetic acid at room temperature afforded 5,7,4'-trihydroxy-3'-nitroisoflavone **7** in good yield, while its 4'-*O*-methyl ether, biochanin A **6,** was unreactive at room temperature and required treatment with HNO_3 in acetic acid at 70 °C for 7 hrs (Scheme 2) to give 5,7-dihydroxy-4'-methoxy-8-nitroisoflavone **8** in 57% yield. The experimental results show that the order of reactivity in the nitration of genistein **5** is 3' (5') > 8 > 6. In the case of Biochanin A **6** the experimentally observed order of reactivity in nitration is 8 > 3' > 6 > 5'. The reduction

of the nitroisoflavones **4, 7** and **8** to aminoisoflavones **5**, **9**, and **10** was best carried out using an excess of tin (II) chloride dihydrate under nitrogen in 78-97% yield. The work-up has also to be carried out under a nitrogen atmosphere.



5 (R_1 = H)
6 (R_1 = Me)

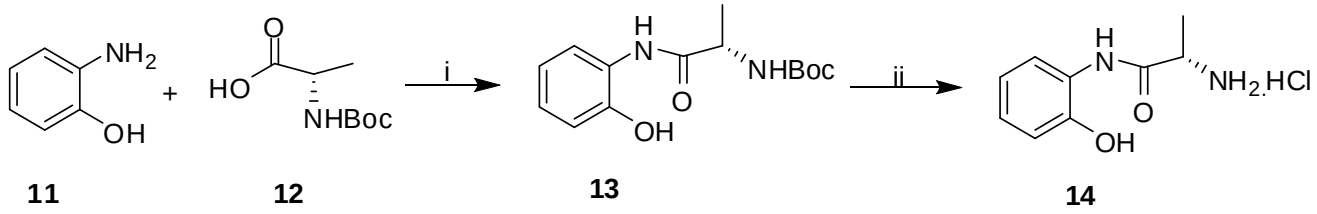
7 (R_1 = H, R_2 = NO_2, R_3 = H; 94%)
8 (R_1 = Me, R_2 = H, R_3 = NO_2; 54%)

9 (R_1 = H, R_2 = NH_2, R_3 = H; 97%)
10 (R_1 = Me, R_2 = H, R_3 = NH_2; 95%)

Scheme 2. i) HNO_3, acetic acid; ii) $SnCl_2.2H_2O$, EtOH, 70-75 ^oC.

3.3. Coupling to L-alanine

The three aminoisoflavones, **5**, **9** and **10**, were to be coupled with *N*-Boc-L-alanine without further purification, due to their instability, using standard peptide coupling methods.To explore methods for coupling of catechol-type aminophenols with *N*-Boc-L-alanine, we first turned our attention to the preparation of *S*-2-amino-*N*-(2-hydroxyphenyl) propionamide **13** as a model compound (Scheme 3) using commercially available *o*-aminophenol **11**. The coupling reaction of aminophenol with *N*-Boc-L-alanine **12** was carried out in the presence of DCC/HOBt.H_2O in dry DMF under nitrogen at rt. Removal of the Boc protecting group was performed by stirring **13** in 3N HCl-dioxane to successfully yield the hydrochloride salt of *S*-2-amino-*N*-(2-hydroxyphenyl) propionamide **14** (Scheme 3).



11 **12** **13** **14**

Scheme 3. i) DCC, HOBt.H_2O, dry DMF (61%); ii) 6 N HCl-dioxane, dioxane (86%).

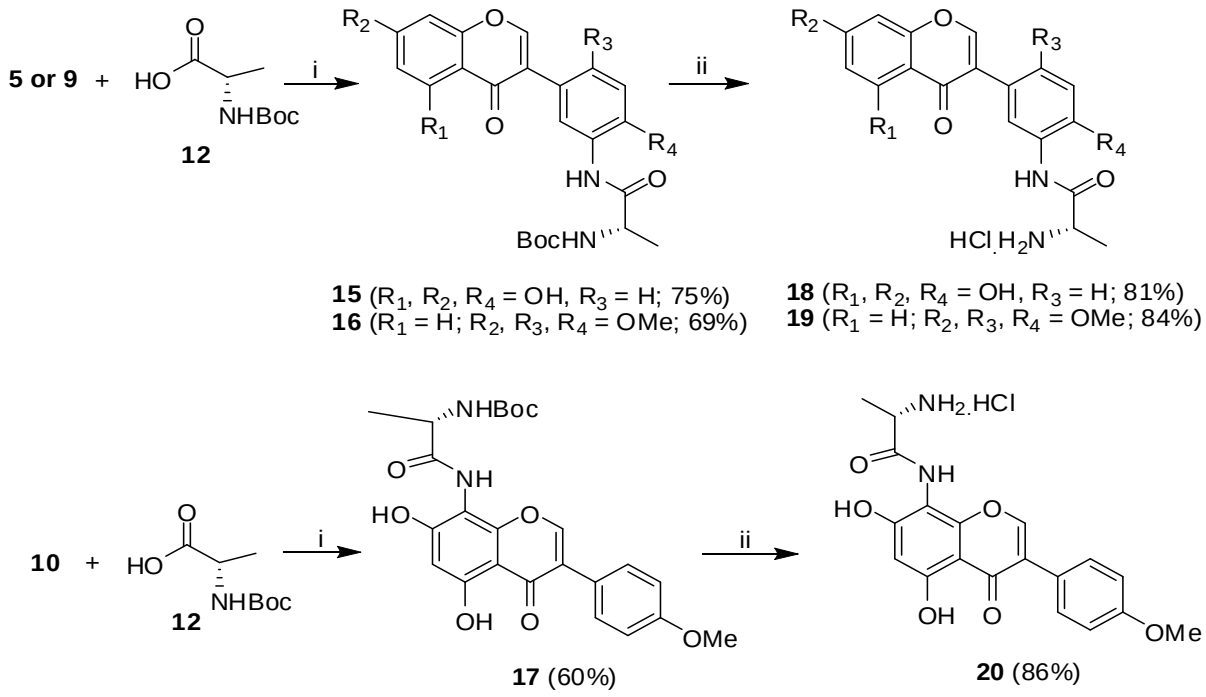
The above described procedure was then successfully adapted for the synthesis of S-2-amino-*N*-isoflavonyl propionamides **15-17.** Treatment of the aminoisoflavones with *N*-Boc-L-alanine in the presence of DCC/HOBt.H_2O in dry DMF, followed by removal of the Boc protecting group with 3N HCl afforded the target compounds **18-20** in good yields and purity (Scheme 4).

3.4. Biological evaluation of L-alanyl aminoisoflavone conjugates

Table 1 showed the evaluation which was carried out using compounds **18, 19** and **20** at 1 mM concentration in an agar medium (Micro agar CAS 9002-18-0, Batch 000137.03). Two strains were grown on these plates namely *Escherichia coli* and *Staphylococcus aureus*, however there were no apparent color changes on the plates as the bacteria were grown at 37 ^oC.

A second batch of experiments was carried out using four control strains of bacteria, namely *E. coli*, *E. coli* ATCC 25923 and *S. aureus* ATCC 25922, and the test compounds were included in the media at 10 mM concentration and in presence of ferric ammonium sulfate (0.05 mM). Two different media were

employed. These were Agar CM0337 Mueller-Hinton Agar, and CM0001 Nutrient Broth containing Micro agar CAS 9002-18-0, Batch 000137.03.



Scheme 4. i) DCC, HOBt.H_2O, dry DMF; ii) 6 N HCl-dioxane, dioxane.

Table 1. Results from growth of bacteria on media containing test compounds.

Test Compound and Media	***E. coli* ATCC 25923**	***S. aureus* ATCC 25922**
14 in CM0337	Black Colonies	Light yellow colonies
14 in CM0001	Black Colonies	Light yellow colonies
18 in CM0337	Creamy colonies	Creamy colonies
18 in CM0001	Creamy colonies	No colonies
19 in CM0337	Creamy colonies	Creamy colonies
19 in CM0001	Creamy colonies	Creamy colonies
20 in CM0337	Creamy colonies	Creamy colonies
20 in CM0001	Creamy colonies	Creamy colonies

The experiments with on the various doped agar media were generally negative with no indication of black colonies resulting from enzymic hydrolysis of the conjugates and subsequent decomposition of the aminoflavone. However black colonies were observed with the *o*-aminophenol conjugate but only in the presence of the two *E. coli* strains and not the *S. aureus*. It was also noted that bacterial growth was poorer in the presence of the isoflavone conjugates, in particular the genistein derivative, possibly due to some anti-bacterial action.

Conclusion

In conclusion it can be seen that three novel aminoisoflavones have been prepared *via* nitration of the parent isoflavone and reduction using tin (II) chloride dihydrate. These unstable aminoisoflavones were then coupled with L-alanine, to give stable L-alanyl aminoisoflavone conjugates which were successfully synthesized in good yields. *S*-2-Amino-*N*-(2-hydroxyphenyl) propionamide was also prepared. The biological evaluation of the compounds only showed positive results for the *o*-aminophenol conjugate which gave black colonies with the *E. coli* strains on agar plates. From these preliminary experiments it is not clear why the compounds did not produce

black colonies. It could simply be that they are not substrates for the L-alanyl aminopeptidase, or else that they are not taken up into the bacterial cells during growth. Further experiments using isolated L-alanyl aminopeptidase would shed further light on this problem.

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Conflicts of Interest: The authors declare no conflict of interest.
Data Availability: The data used to support the findings of this study are available from the corresponding author upon request.

References

1. F.H. Sarkar, Y. Li, Z. Wang, S. Padhye, Lesson learned from nature for the development of novel anti-cancer agents: implication of isoflavone, curcumin, and their synthetic analogs, *Curr. Pharm. Des.*, 16(16) (2010) 1801-1812. https://doi:10.2174/138161210791208956
2. W. Szeja, G. Grynkiewicz, A. Rusin, Isoflavones, their glycosides and glycoconjugates. Synthesis and biological activity, *Curr. Org. Chem.*, 21(3) (2017) 218-235. doi: 10.2174/1385272820666160928120822
3. M. Cellier, A.L. James, S. Orenga, J.D. Perry, G. Turnbull, S.P. Stanforth, The synthesis of L-alanyl and *β*-alanyl derivatives of 2-aminoacridone and their application in the detection of clinically important microorganisms, PLoS ONE, 11(7) (2016): e0158378. https://doi:10.1371/journal.pone.0158378.
4. C. Gao, I. Uzelac, J. Gottfries, L.A. Eriksson, Exploration of multiple sortase A protein conformations in virtual screening, *Sci. Rep*., 6 (2016) 20413. https://doi: 10.1038/srep20413
5. M. Manafi, W. Kneifel, Rapid methods for differentiating Gram- positive from Gram- negative aerobic and facultative anaerobic bacteria, *J. Appl. Bacteriol.*, 69 (1990) 822-827. https://doi.org/10.1111/j.1365-2672.1990.tb01579.x
6. D.F. Liu, C.C. Cheng, A facile and practical preparation of 5,7- dihydroxy- 3- (4- nitrophenyl)- 4*H*- 1-benzopyran- 4- one, *J. Heterocycl. Chem.*, 28(6) (1991) 1641-1642. doi.org/10.1002/jhet.5570280632
7. S.S. Karmarkar, Synthetical experiments in the chromone group: Part XXXDI-further applications of the ethyl orthoformate method for the synthesis of isoflavones, *J. Sci. Ind. Research (India)*, 20B (1961) 234-238.
8. V.P. Khilya, M.S. Luk'yanchikov, A.L. Kazakov, N.V. Gorbulenko, Synthetic analogs of natural isoflavones; *Ukrainski Khimicheskii Zhurnal* (Russian) 50 (1984) 1301-1306.
9. O.V. Singh, R.S.A. Kapil, General method for the synthesis of isoflavones by oxidative rearrangement of flavanones using thallium **(III)** perchlorate, *Indian J. Chem.*, 32B (1993) 911-915.
10. N.K. Chudgar, N.V. Mani, S. Sethna, Studies in isoflavones-Part I. Iodination, bromination and nitration of 6-hydroxyisoflavone and its methyl ether, *J. Inst. Chem. (India)*, 42 (1970) 194-198.
11. N.K. Chudgar, N.V. Mani, S. Sethna, Studies in isoflavones. I. Bromination, iodination, and nitration of 7-hydroxyisoflavone, *J. Inst. Chem.(India)* 39 (1967) 203-208.
12. N.V. Mani, S. Sethna, Studies in isoflavones-Part ID. Nitration of 7,8- and 6,7-dihydroxyisoflavone and their methyl ether, *J. lnst. Chem., Calcutta*, 43 (1971) 234-240.
13. S. Balasubramanian and M.G. Nair, An efficient "One Pot" synthesis of isoflavones, *Synth. Commun.*, 30 (2000) 469-484. https://doi.org/10.1080/00397910008087343
14. J.L. Bose, N.L. Dutta, lsoflavones: Part II- a new synthesis of isoformononetin and daidzein. *J. Sci. Ind. Research* (Indian), 17B (1958) 266-268.
15. D.F. Diedrich, T.A. Scahill, S.L. Smith, Preparation and physical properties of some deoxybenzoins and isoflavones, *J. Chem. Eng. Data*, 22 (1977) 448-451. https://doi.org/10.1021/je60075a021
16. F.D. Bellamy, K. Ou, Selective reduction of aromatic nitro compounds with stannous chloride in non acidic and non aqueous medium, *Tetrahedron Lett.*, 25(8) (1984) 839-842. https://doi.org/10.1016/S0040-4039(01)80041-1
17. A. Pelter, R.S. Ward, J.L. Whalley, A facile synthesis of 2-substituted isoflavones for immunoassay: assembly of the isoflavonoid skeleton by means of a novel cyclization reaction, *Synthesis*, 12 (1998) 1793-1802. https://doi: 10.1055/s-1998-2221