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7-Chloro-4-methyl-6-nitro-2*H*-chromen-2-one: a novel type of reagent for fluorescence analysis

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Abstract—Starting from 7-chloro-4-methyl-2*H*-chromen-2-one **1** we have obtained a new reagent for fluorescence analysis. The synthesised key compound 7-chloro-4-methyl-6-nitro-2*H*-chromen-2-one **2** forms highly fluorescent derivatives with different amines, which can be introduced by nucleophilic substitution of the chloro atom. Two examples are given which describe the preparation of these derivatives. Additionally, we demonstrate that an increase of fluorescence can be achieved, if a reduction step is performed after the substitution.

It has been shown that—depending on their substitution pattern—derivatives of 2*H*-chromen-2-one may exhibit high fluorescence activity and therefore are useful candidates for dyes, fluorescent substrates in enzymatic assays or derivatisation reagents in analytical assays.[1] With the latter application, derivatisation has been hitherto carried out via amidation, transesterification, esterification, reaction with isothiocyanates or by condensation.

In the present paper we present 7-chloro-4-methyl-6-nitro-2*H*-chromen-2-one **2**, a compound, which—although itself exhibiting no fluorescence—nevertheless is a versatile derivatisation reagent for analysis using fluorescence detection. Nucleophilic aromatic substitution has been-since Sanger[2]—an established reaction for derivatisation of analytes. It allows reagent attachment at nitrogen, oxygen and sulfur atoms. The spatial distance between reagent and analyte is minimised, when compared to other methods of attachment (e.g. via carboxyl groups: esters, amides, carbamates, ureas). Due to the fact that by this reaction the analyte is linked to the chromophore of the reagent via a heteroatom bearing a free electron pair, it may be expected that specific changes in the spectroscopic properties might occur. Apart from an impact on absorption wavelengths or extinction coefficients of the reagent, also effects on chiroptical properties (e.g. enhancement of optical rotation values) of the analyte are to be expected. Due to its character as an aromatic second order substituent, the nitro group is required to achieve nucleophilic aromatic substitution. It may be reduced after the reaction to enhance fluorescence. The resulting amino group does not only have a positive impact on fluorescence properties of the compound, but also provides, if intended, an additional position for attachment of analytes.

Although 2*H*-chromen-2-ones (coumarins) are compounds which are known for more than 100 years,[3] the methods reported for their synthesis require major modifications to obtain compound **2** in acceptable yields. Therefore an efficient synthesis of compound **2** will be reported in the present paper together with one example to illustrate the synthetic procedure for nucleophilic substitution and reduction of the nitro group and a second example, by which the impact of the introduction of the reagent on the optical rotation of L-prolinol is demonstrated.

Synthesis of compound 2

A significant portion of published 2*H*-chromen-2-ones bear a 4-methyl group. Such compounds with halogen or nitro groups at the aromatic nucleus may be synthesised according to the procedure published by Clayton.[4] He describes a modification of the Pechmann coumarin synthesis, by which a phenolic compound reacts with a β-keto ester derivative. In this reaction the substituent at the *meta*-position of the phenol used in the reaction will become the substituent at position 7 of the coumarin. It has also been reported that an amino group

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increases fluorescence of coumarins. Amino groups have been introduced by nitration followed by a reduction step. Following this synthetic approach several derivatives of aminoumbelliferones[5] have been synthesised. However, the 7-chloro-6-nitro derivative **2** has not yet been described.

We obtained compound **2** by selective nitration of 7-chloro-4-methyl-2*H*-chromen-2-one **1**, which was synthesised according to the procedure of Brubaker et al., who describes a modification of the Pechmann condensation leading to improved, but still moderate yields and increased purity of the product.[6] This protocol could be reproduced, leading to compound **1** in 30% yield.

Different methods have been reported describing the nitration of closely related compounds of the coumarin type.[7] In general, mixtures of mono and dinitro products resulted in almost equal amounts and were separated by chromatographic methods or recrystallisation.[7,8] In order to avoid these impurities and to achieve higher yields, reaction conditions were modified as follows: first, we applied concentrated nitric acid both as solvent and reagent for nitration. Compound **1** (1.33 g, 6.8 mmol) was dissolved in 30 ml of concentrated nitric acid keeping the solution constantly below −5°C for 2 h. After that time the starting compound had reacted quantitatively and only one product was detected by TLC. The mixture was treated with crushed ice and the precipitated product isolated by suction filtration. Recrystallisation from ethanol yielded 2.5 g of pure product. NMR spectroscopy showed that the isolated product was neither the desired mononitro compound **2** nor the 6,8-dinitro compound **2a**, a substitution pattern which has been described in literature for related coumarins,[5,7,8] but was 7-chloro-3,6-dinitro-4-methyl-2*H*-chromen-2-one **2b** (Fig. 1).

Structure and substitution patterns of this compound were confirmed by ^{13}C and ^{1}H NMR analyses, mass spectroscopy and elemental analyses.[9] Monitoring of the reaction mixture by TLC showed that during the reaction other products could be observed only in traceable amounts. The formation of the undesired dinitro product was probably caused by the excess of nitric acid applied. To avoid this, the reaction was carried out using concentrated sulphuric acid as solvent (50 ml for 10 g **1**), adding a mixture of concentrated sulphuric (5.2 ml) acid and nitric acid (4.3 ml) below 0°C, yielding 7-chloro-6-nitro-4-methyl-2*H*-chromen-2-one in a yield of more than 90% after 1 h of stirring. The removal of side products, particularly traces of 7-chloro-3,6-dinitro-4-methyl-2*H*-chromen-2-one **2b**, which had been observed by TLC, could be achieved by recrystallisation from toluene.[10]

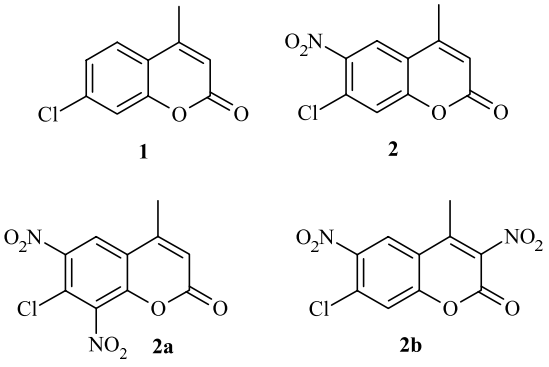


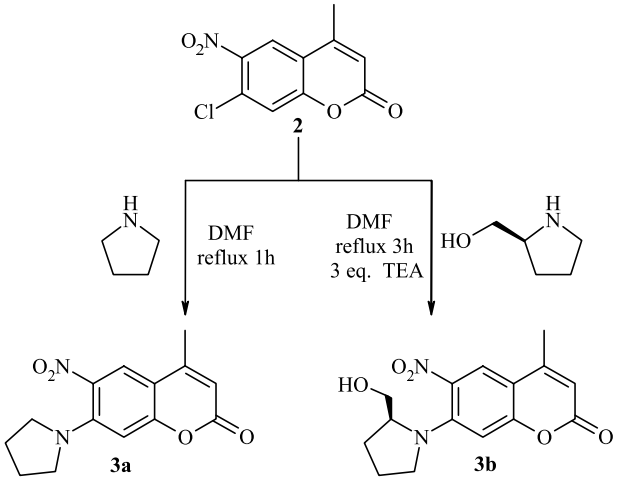
Figure 1.

Nucleophilic substitution of 2 and reduction of the nitro group

Nucleophilic substitution was carried out using the secondary amines pyrrolidine and L-prolinol as substrates. Thus, compound **3a** was synthesised by dissolving 1 g (4.2 mmol) of **2** in 20 ml of DMF, adding 0.65 g (9.2 mmol) of pyrrolidine and heating the reaction mixture to reflux temperature for 1 h under an argon atmosphere. After cooling to room temperature, the mixture was treated with crushed ice, the precipitate was filtered off and recrystallised from methanol to yield 0.72 g (63%) of **3a**.[11,12] Applying the same conditions, 1 g (4.2 mmol) of **2** and 0.94 g (9.2 mmol) of L-prolinol yielded 0.9 g (78.9%) of **3b**. Considering the fact that one molar equivalent of L-prolinol was consumed in the reaction as basic scavenger, we decided to attempt the use of triethylamine for this purpose. Thus, 6 g (25.2 mmol) of **2** were dissolved in 120 ml DMF and 7.8 g of triethylamine (77.5 mmol). After adding 2.82 g (27.6 mmol) of L-prolinol the mixture was heated to reflux for 3 h under an argon atmosphere and worked up as described for compound **3a**. The modification afforded 6.28 g (81.7%) of **3b**.[13]

Therefore, addition of triethylamine seems to be recommendable, particularly in cases where the amines are not readily available (Scheme 1).

Three different methods for reduction of the nitro group were evaluated: $SnCl_2$/HCl,[14] sodium dithionite/NH_3[15] and $SnCl_2$/ethanol.[16] The method using $SnCl_2$ in ethanol without adding acid proved to be the most



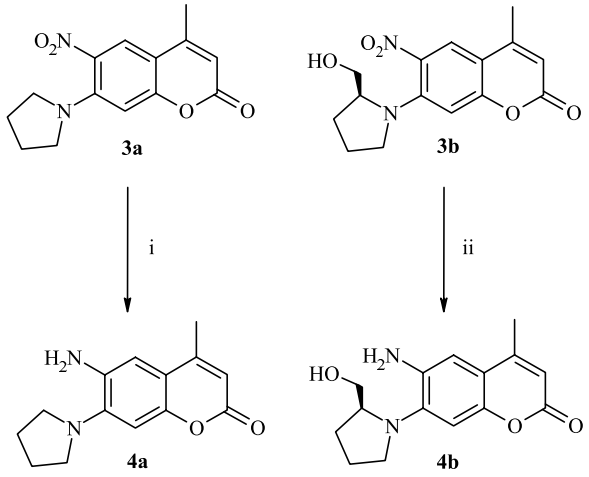
Scheme 1. Nucleophilic substitution.

suitable. Compound **4a** was prepared using $SnCl_2$/HCl in ethanol for the reduction of **3a**. This procedure gave 72% of a crude product, which after purification by VFC[17] afforded 36% of **4a**[18] as pure product. Reacting compound **3b** with $SnCl_2$ in ethanol at 60°C without addition of acid yielded the desired compound **4b**,[19] which was recrystallised from methanol and isolated in nearly quantitative yield. At this point it seems worth mentioning that compounds **4a** and **4b** are the first 6,7-diamino substituted coumarins hitherto described (Scheme 2).

The resulting purified amines were dissolved and fluorescence spectra were measured. In Figure 2 the fluorescence spectrum of **4b** is shown. The substance shows high fluorescence, its excitation maxima depend on both pH and type of solvent. Compared to the unreduced nitro-derivative, an approximately 100-fold increase of fluorescence was observed.[20]

Effect of the 'coumarinisation' on optical rotation values of L-prolinol

Comparing the values of optical rotation of L-prolinol[21] with the 'coumarinised' nitro compound **3b** and the corresponding amino compound **4b** revealed that much higher optical rotation values were obtained after the nucleophilic substitution reaction.



Scheme 2. *Reagents and conditions*: (i) $SnCl_2$/conc. HCl/EtOH, 6 h, rt; (ii) $SnCl_2 \times 2H_2O$/EtOH, 1 h, reflux.

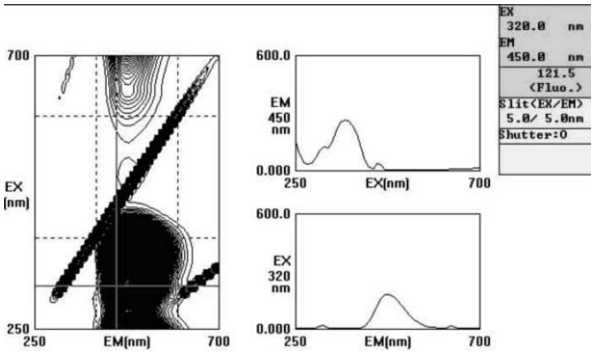


Figure 2. Fluorescence spectra of **4b** in dichloromethane (10 μg/ml), EM: 250–700 nm, EX: 250–700 nm.

In conclusion, the coumarin **2** has been described for the first time. A method for its synthesis has been presented. Nucleophilic aromatic substitution allows attachment of an amine. Reduction of the nitro group leads to hitherto not described 6,7-diamino substituted 4-methyl-coumarins, which are highly fluorescent compounds useful in fluorescence analysis. The loss in fluorescence was remarkably less pronounced, compared to other dyes, like NBD,[22] if water was used as solvent. Even though the fluorescence does not come up to that of fluorescamine, the limit of detection is almost similar to the one, which is obtained with dyes of the naphtholsulfonic acid-type.[23]

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9. Analytical data for compound **2b**: yellow crystals; mp 205–207°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.74 [s, 1H]; 8.14 [s, 1H], 2.53 [s, 3H]; ^{13}C NMR (50 MHz, DMSO-d_6): δ 153.3 [C_2=O], 152.1 [C_9(Ar)], 144.9 [C_6(Ar)], 144.4 [C_4], 137.8 [C_3], 130 [C_7(Ar)], 124.9 [C_5(Ar)], 120.1 [C_8(Ar)], 117.9 [C_{10}(Ar)], 14.1 [CH_3]; IR (KBr): 3113, 3051, 1742, 1555, 1347, 1041 cm^{-1}; MS: m/z (ES-): 282.9. Anal. calcd for $C_{10}H_5ClN_2O_6$: (284.6): C, 42.2; H, 1.77; N, 9.84. Found: C, 42.18; H, 1.85; N, 9.48%.
10. Analytical data for compound **2**: white crystals; mp 255°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.48 [s, 1H]; 7.94 [s, 1H], 6.6 [s, 1H], 2.46 [s, 3H]; ^{13}C NMR (50 MHz, DMSO-d_6): δ 158.3 [C_2=O], 154.7 [C_9(Ar)], 151.9 [C_4], 143.6 [C_6(Ar)], 128.0 [C_7(Ar)], 123.1 [C_5(Ar)], 119.5 [C_8(Ar)], 119.3 [C_{10}(Ar)], 116.1 [C_3], 17.9 [$C_{11}H_3$]; MS: m/z (ES-): 239.0. Anal. calcd for $C_{10}H_6ClNO_4$: (239.62): C, 50.13; H, 2.52; N, 5.85. Found: C, 50.07; H, 2.72; N, 5.88%.
11. Analytical data for compound **3a**: yellow needles; mp 167–168°C; ^{1}H NMR (90 MHz, $CDCl_3$): δ 7.94 [s, 1H]; 6.67 [s, 1H], 6.05 [s, 1H], 3.39–3.14 [t, 4H], 2.36 [s, 3H], 2.11–1.96 [m, 4H]; IR (KBr): 1740, 1530, 1390, 1290 cm^{-1}. Anal. calcd for $C_{14}H_{14}N_2O_4$: (274.28): C, 61.31; H, 5.14; N, 10.21. Found: C, 61.15; H, 5.16; N, 10.32%.
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13. Analytical data for compound **3b**: golden crystals; mp 172–173°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.1 [s, 1H]; 7.0 [s, 1H], 6.18 [s, 1H], 4.86–4.82 [t, 1H], 4.0–3.9 [m,

1H], 3.5 [m, 1H], 3.36 [m, 2H], 2.8 [m, 1H], 2.3 [s, 3H], 1.9–1.6 [m, 4H]; ^{13}C NMR (50 MHz, DMSO-d_6): δ 159.8 [*C*=O], 155.7 [C_9(Ar)], 153.1 [C_4], 144.3 [C_6(Ar)], 135.0 [C_7(Ar)], 124.3 [C_5(Ar)], 110.9 [C_3], 109.4 [C_{10}(Ar)], 102.9 [C_8(Ar)], 61.7 [$C_{6'}$], 60.9 [$C_{2'}$], 52.6 [$C_{5'}$], 28.6 [$C_{3'}$], 24.4 [$C_{4'}$], 17.9 [$C_{11}H_3$]; MS: *m*/*z* (ES+): 305.1. Anal. calcd for $C_{15}H_{16}N_2O_5$: (304.31): C, 59.21; H, 5.3; N, 9.21. Found: C, 58.97; H, 5.36; N, 9.11%. $[\alpha]^{20}_{546}$: −1995; $[\alpha]^{20}_{589}$: −1354 (*c* 1.0, DCM).

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17. Vacuum-flash-chromatography.
18. Analytical data for compound **4a**: bright yellow crystals; mp 164–166°C; ^{1}H NMR (90 MHz, $CDCl_3$): δ 6.81 [s, 2H]; 6.08 [s, 1H], 3.66 [s, 2H], 3.26–3.07 [t, 4H], 2.33 [s, 3H], 2.08–1.82 [m, 4H]; IR (KBr): 3380, 3300, 1690 cm^{-1}. Anal. calcd for $C_{14}H_{16}N_2O_2$: (244.29): C, 68.83; H, 6.6; N, 11.47. Found: C, 68.72; H, 6.62; N, 11.41%.
19. Analytical data for compound **4b**: green–yellow crystals; mp 256–259°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.87 [s, 1H]; 6.8 [s, 1H], 6.0 [s, 1H], 4.75 [s, 2H], 4.46–4.42 [t, 1H], 3.8 [bs, 1H], 3.5 [m, 1H], 3.26–3.11 [m, 2H], 2.77 [m, 1H], 2.26 [s, 3H], 2.0–1.7 [m, 4H]; ^{13}C NMR (50 MHz, DMSO-d_6): δ 160.8 [*C*=O], 152.8 [C_4], 146.5 [C_9(Ar)], 141.4 [C_6(Ar)], 139.0 [C_7(Ar)], 113.5 [C_{10}(Ar)], 110.7 [C_3], 108.2 [C_5(Ar)], 105.3 [C_8(Ar)], 62.4 [$C_{6'}$], 60.0 [$C_{2'}$], 51.3 [$C_{5'}$], 27.9 [$C_{3'}$], 23.6 [$C_{4'}$], 18.1 [CH_3]; MS: *m*/*z* (ES+): 275.1. Anal. calcd for $C_{10}H_7NO_5$: (274.32): C, 64.41; H, 6.70; N, 10.01. Found: C, 64.13; H, 6.82; N, 9.36%. $[\alpha]^{20}_{546}$: +169; $[\alpha]^{20}_{589}$: +125 (*c* 1.0, DCM).
20. Fluorescence was measured and quantified at three different wavelengths both for excitation and emission: EX 350/EM 500 nm; EX 350/EM 580 nm; EX 325/EM 520 nm.
21. L-Prolinol $[\alpha]^{20}_{546}$: +37 (*c* 1.0, toluene).
22. 7-Nitrobenz-2-oxa-1,3-diazole.
23. The compounds **2**, **3b** and **4b** are commercially available from Prochem KFT (prochem@chello.at).