SYNTHESIS OF o-ACYLAMINO-4-METHYL-7-HYDROXYCOUMARINS (4-METHYLUMBELLIFERONES)

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Some acylated o-amino-7-hydroxycoumarins have been obtained which may be used as intermediates in the preparation of fluorogenic substrates for certain enzymes.

This communication describes the synthesis of 6- (I) and 8-N-hexadecanoylamino-4-methylumbelliferone (II), the aglycones of the corresponding glycosides, which can be used as fluorogenic substrates for the measurement of the activity of glycocerebrosidases, and in the diagnosis of certain hereditary diseases which develop as a result of deficiencies of these enzymes in the human body.

There has in recent years been increasing use of fluorescent-tagged analogs of the natural substrates of glycocerebrosidases in the measurement of the activity of these enzymes, but the isolation and modification of the natural substrates is a laborious and tedious process which has retarded considerably their application in biochemical diagnostics [1].

Synthetic substrates are also known which can be used in measuring the activity of cerebrosidases, such as 2-hexadecanoylamino-4-nitrophenyl-β-O-galactopyranoside [2] and -β-O-glucopyranoside [3], but despite their undoubted advantages over the semisynthetic materials (simplicity of preparation, accessibility, and thermal stability) they have serious disadvantages, namely, insufficient specificity with respect to the enzyme [4] and the low sensitivity of the method for the measurement of the activity of the enzyme as compared with fluorogenic and, even more so, radioactive methods.

The development of a method for the synthesis of fluorogenic substrates of a glycolipid type would combine the advantages of synthetic substrates (accessibility and stability) with high sensitivity of measurement of enzyme activity. No such fluorogenic substrates have not yet been reported, however, nor are the aglycones required for their synthesis known. A solution of this problem (the synthesis of fluorogenic aglycones and examination of their physicochemical properties) is reported here.

The starting material for the synthesis of the aglycones (I) and (II) was 4-methylumbelliferone (III).

The key feature of the synthesis was the choice of conditions for nitration which enabled either the 6- or the 8-nitro-isomer of (III) to be obtained selectively. Examination of the literature on the nitration of (III) led to the conclusion that the dominant factor influencing the course of the reaction was the acidity of the medium. For example, in the nitration of (III) in conc. sulfuric acid, 58% of nitro-products were obtained in which the ratio of (IV) to (VII) was 1:2, whereas in glacial acetic acid the total yield of nitration products was 46% and the ratio of (IV) to (VII) was 3.5:1, i.e., initial protonation of (III) under the nitration conditions appears to favor the formation of the 8-nitro-isomer, whereas nitration without prior protonation affords the 6-isomer. It may be assumed that nitration with a nitronium salt in an aprotic solvent [6] would give predominantly the 6-nitro-isomer.

It was found that the nitration of (III) with nitronium tetrafluoroborate in acetonitrile gave (IV) in 65% yield. According to TLC, the 8-nitro-isomer was present in the reaction mixture in trace amounts only.

The reduction of the 8-nitro-isomer (VII) with sodium hydrosulfite in aqueous ammonia has been described [7]. We have found that reduction of (IV) to the 6-amino-compound (V) also affords qualitative yields under similar conditions.

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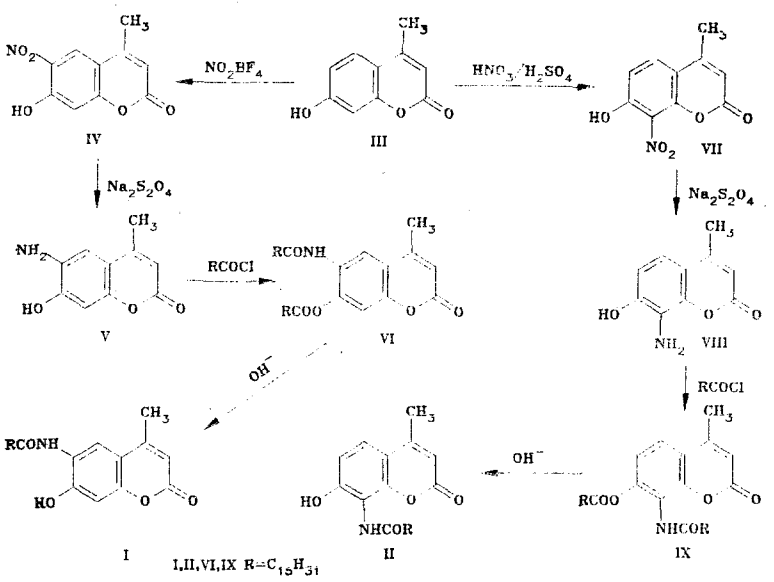
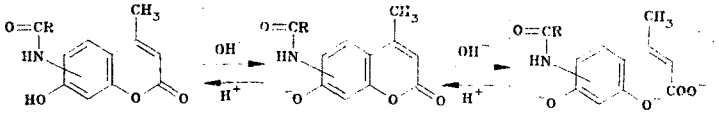


Acylation of the o-aminohydroxycoumarins (V) and (VIII) with palmitoyl chloride was carried out by the method normally used for the selective acylation of the amino-group in o-aminophenols, namely, by reaction of the acyl chloride with aminophenol in pyridine [2]. Unlike 2-amino-4-nitrophenol, which is acylated in this way at the amino-group only, however, (V) underwent acylation at both the amino- and hydroxy-groups. In the present case, the product was the diacyl derivative (VI). The maximum yield of (VI) (98%) was obtained when the ratio of amine (V) to acid chloride was 1:2. When the reactant ratio was 1:1, the yield of (VI) was around 50%, and the reaction mixture contained unreacted amine (V). No monoacylation product was found. The acylation of (VIII) proceeded similarly to give the diacyl derivative.

The final stage in the synthesis of the aglycones, hydrolysis of the ester group in (VI) and (IX), was effected by treatment of the latter with 5% alcoholic KOH followed by acidification of the reaction mixture with HCl to pH 5-6. The overall yields of (I) and (II) were 60 and 30% respectively, calculated on 4-methylumbelliferone.

The structures of the compounds were established by their UV, IR, and PMR spectra, and confirmed by their elemental analyses (Table 1).

The IR spectra of (I), (II), (VI), and (IX) showed absorption of the NH group at 3275-3335 cm^{-1}.



Compounds (I) and (II) fluoresce in solution, the type of fluorescence being similar to that described for (III) [8]. The fluorescence was enhanced on basification of the solution, reaching a maximum a pH 10-11, i.e., when the aglycone was converted from the nonionized state to the ionic "phenoxide" form. Addition of more than an equimolar amount of the basic reagent resulted in the disappearance of the fluorescence, apparently as a result of cleavage of the pyrone ring, which recyclizes on acidification of the solution.

Table 2 shows the absorption and fluorescence spectral data, together with the quantum yields of fluorescence of compounds (I-III). The magnitude of the quantum yield in o-amino-acyl-4-methylumbelliferones is determined by the position of the acylamino-group relative to the pyrone ring. The introduction of the acylamino-substituent into the 6-position has little effect on the quantum yield, whereas in the 8-substituted derivative, in which the substituent is located immediately adjacent to the pyrone ring, the quantum yield is lower by a factor of 65 than in unsubstituted (III).

Of the two o-acylamino-4-methylumbelliferones synthesized, therefore, (I) has greatly superior fluorogenic properties, and is recommended for the synthesis of fluorogenic substrates as the aglycone.

Table 1. Constants of Compounds (I), (II), (VI), and (IX)

Compound	mp, °C	R_f (B)	PPM spectrum, δ, ppm	Found, %		Empirical formula	Calculated, %	
				C	H		C	H
I	191—194	0,65	0,8 (3H, t, CH_3-aliph.); 1,15 (24H, s, CH_2-aliph. γ...); 1,35 (2H, m, β-CH_2-aliph.); 2,31 (3H, s, CH_3arom.); 2,35 (2H, m, CH_2—C=O); 6,12 (1H, s, H—C_3); 6,81 (1H, s, H—C_8); 8,17 (1H, s, H—C_5); 9,35 (1H, s, NH)	72,8	9,2	$C_{26}H_{39}NO_4$	72,7	9,1
II	135—136	0,90	0,81 (3H, t, CH_3-aliph.); 1,17 (24H, s, CH_2-aliph. γ...); 1,35 (2H, m, CH_2-arom. β); 2,31 (3H, s, CH_3-arom.); 2,35 (2H, m, CH_2—C=O); 6,07 (1H, s, H—C_3); 6,87 (1H, d, H—C_6, J=9,5 Hz); 7,45 (1H d, H—C_5, J=9,5 Hz)	73,0	8,9	$C_{26}H_{39}NO_4$	72,7	9,1
VI	132—133	0,60	0,81 (6H, t, $2CH_3$-aliph.); 1,18 (48H, s, CH_2-aliph.); 2,35 (3H, s, CH_3-arom.); 6,21 (1H, s, H—C_3); 7,13 (1H, s, H—C_8); 8,42 (1H, s, H—C_5); 9,40 (1H, s, NH)	75,4	10,0	$C_{42}H_{69}NO_5$	75,6	10,3
IX	116—118	0,90	0,84 (6H, $2CH_3$-aliph.); 1,21 (48H, s, CH_2-aliph.); 1,62 (4H, m, $2CH_2$-β); 2,40 (3H, c, CH_3-arom.); 2,50 (4H, m, $2CH_3$—C=O); 6,23 (1H, s, H—C_3); 7,18 (1H, d, H—C_6, J=9,6 Hz); 7,65 (1H, d, H—C_5, J=9,60 Hz); 9,40 (1H, s, NH)	75,7	10,8	$C_{42}H_{69}NO_5$	75,6	10,3

Table 2. UV and Fluorescence Spectra of (I-III), (VI), and (IX)

Compound	UV spectrum, λ_{max}, nm (ε·10^3)		Fluorescence spectrum†				
			neutral form			anionic form	
	neutral form	anion form *	λ_{exc}	λ_{em}	$Q\times10^{-2}$	λ_{exc}	λ_{em}
I	340 (14,7)	385 (30,0)	342	415	27	382	450
II	320 (8,0)	369 (19,8)	330	430	0,458	370	480
III‡	315 (13,6)	360 (17,6)	317	380	32,5	365	448
VI	320 (6,7)						
IX	282 (7,6)						

*Spectra obtained at pH 10.5 (ethanol—aqueous buffer, 5:2).
†Spectra uncorrected; λ_{exc} — λ_{max} excitation, λ_{em} — λ_{max} emission of fluorescence; Q is the quantum yield of fluorescence.
‡Data from [8].

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EXPERIMENTAL

PMR spectra were obtained on a Bruker WH-250 spectrometer [in $(CD_3)_2SO$], UV spectra on a Specord M-40 (in ethanol), and fluorescence spectra on a Hitachi MPF 4A spectrofluorimeter (in ethanol). TLC was carried out on Silufol-254 plates in the systems ethanol—chloroform, 1:15 (A), ethyl acetate (B), and ethyl acetate—chloroform, 1:2 (C).

6-Nitro-4-methylumbelliferone (IV). To a suspension of 5 g (28.4 mmole) of (III) in 50 ml of dry acetonitrile was added in small portions with stirring at −10°C 3.8 g (28.5 mmole) of nitronium tetrafluoroborate, and the mixture stirred for 30 min at 0°C. The solid was filtered off, and washed with acetonitrile and ether to give 3 g (65%) of (IV), mp 257-260°C. Mixed mp with a sample obtained as described in [5], 258°C. R_f 0.5 (A).

8-Nitro-4-methylumbelliferone (VII) was obtained as described in [5]. R_f 0.2 (A).

6-Amino-4-methylumbelliferone (V). To a suspension of 5.5 g (25 mmole) of (IV) in 30 ml of 25% aqueous ammonia was added at 20°C 35 ml of 15% aqueous $Na_2S_2O_4$, until the color of the reaction mixture changed from bright orange to light green. The mixture was then boiled for 15 min, cooled to 5°C, and the solid filtered off to give 5.1 g (98%) of (V), mp 272°C. Mp of a mixed sample with material obtained as described in [9], 273°C. R_f 0.65 (B).

8-Amino-4-methylumbelliferone (VIII) was obtained as described in [7]. R_f 0.86 (B).

6-N,7-O-Dihexadecanoyl-6-amino-4-methylumbelliferone (VI). To a solution of 3.3 g (17.3 mmole) of (V) in 90 ml of dry pyridine was added 10.5 ml (35 mmole) of palmitoyl chloride. The mixture was stirred for 3 h at 20°C, 50 ml of acetonitrile added, and the solid filtered off to give 11.3 g (98%) of (VI).

7-O,8-N-Didecanoyl-8-amino-4-methylumbelliferone (IX) was obtained as described for (VI). From 3.3 g (17.3 mmole) of (VIII) there was obtained 10.05 g (87%) of (IX).

6-Hexadecanoylamino-4-methylumbelliferone (I). To a suspension of 1 g (1.5 mmole) of (VI) in 10 ml of ethanol was added with stirring a 5% solution of KOH in ethanol to pH 10-11 (universal indicator), and the resulting solution was treated with stirring with 5% aqueous HCl to pH 5-6. The solid was filtered off, washed well with ethanol and several times with hexane to remove completely any palmitic acid to give 0.6 g (95%) of (I).

8-Hexadecanoylamino-4-methylumbelliferone (II) was synthesized similarly. One gram (1.5 mmole) of (IX) afforded 0.62 g (96%) of (II).

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