ARTICLES

Synthesis of Phenyl 6′-*O*-Malonyl-*β*-D-glucopyranoside. Facile Preparation of Malonylated Glycoconjugates

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Regioselective acylation of glycoconjugates with malonic acid was achieved by using phenyl *β*-D-glucopyranoside as a model glycoside, malonic acid, and *tert*-butyl isocyanide in aprotic solvents. Structural elucidation of phenyl 6′-*O*-malonyl-*β*-D-glucopyranoside was performed by ^{1}H and ^{13}C NMR spectroscopy and high-performance liquid chromatography−atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC−APCI-MS/MS). This one-step reaction opens the way to the preparation of reference substances which are required for the spectroscopic identification of malonylated glycosides in complex natural matrices.

Keywords: *Phenyl 6′-O-malonyl-β-D-glucopyranoside; malonylated glycosides; HPLC−MS/MS; atmospheric pressure chemical ionization (APCI)*

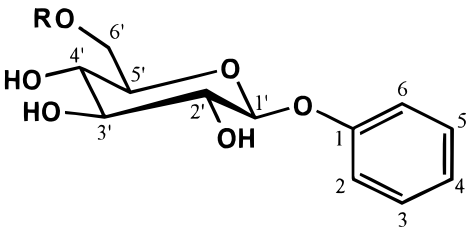
INTRODUCTION

Over the last decade polar *O*- and *C*-glycosylflavonoids, including anthocyanidins, flavones, isoflavones, and flavonols, have been shown to be acylated frequently at the C-6 hydroxyl group of the sugar moiety, e.g., with malonic and succinic acid (Harborne, 1986; Glässgen et al., 1992; Takeda et al., 1993; Wang and Murphy, 1994). As to glycosidically bound flavor compounds (Stahl-Biskup, 1987; Winterhalter and Schreier, 1994), however, reports on malonylated glycosidic flavor precursors are rather scarce to date (Schwab and Schreier, 1988; Moon et al., 1994). This limited information might be caused by chemical and enzymatic degradation processes during isolation procedures (Matern, 1983; Horowitz and Asen, 1989) and, in particular, by the lack of suitable acylated reference glycosides.

Several attempts of malonylation of glycosides have been made. However, enzyme-catalyzed or chemical syntheses were often limited in specificity toward their glycosidic substrates, regioselectivity, and yield or required laborious multistep procedures (Kasai et al., 1981; Matern et al., 1983; Koester et al., 1984; Danieli et al., 1993). Thus, using phenyl *β*-D-glucopyranoside (**1**) as model compound, we developed a convenient one-pot synthesis for the regioselective preparation of 6′-*O*-malonyl glycosides to be used as reference substances, e.g., for on-line LC−MS identification of malonylated glycosides in complex natural matrices.

EXPERIMENTAL PROCEDURES

Materials. Water and methanol, both of HPLC gradient grade, acetic acid, and trifluoroacetic acid (spectroscopic grade) were purchased from Merck (Darmstadt, Germany). *tert*-Butyl isocyanide (^{t}BuNC) and phenyl *β*-D-glucopyranoside (**1**) were obtained from Aldrich (Deisenhofen, Germany). Tetrahydrofuran (THF), acetonitrile, pyridine, and dimethyl sulfoxide (DMSO), all dried over molecular sieves, were purchased from Fluka (Neu-Ulm, Germany).



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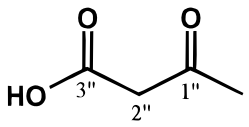

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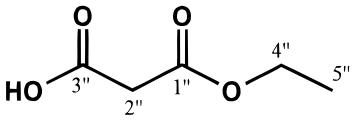

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Synthesis of Phenyl 6′-*O*-Malonyl-*β*-D-glucopyranoside (2). Experiments were performed in 10-mL capsulated flasks or in 1-mL screw-capped vials under argon atmosphere in the dark. Phenyl *β*-D-glucopyranoside and malonic acid were solubilized in dry aprotic solvents (THF, acetonitrile, pyridine, DMSO). *tert*-Butyl isocyanide was added and the mixture was heated for 1 h at 50 °C and stirred for up to 3 days at 25 °C. The reaction was stopped by evaporation of the solvent under reduced pressure. The dry products were dissolved in methanol for subsequent product control and purification using HPLC. Detailed information on various reaction conditions is given in Table 1.

Analysis by High-Pressure Liquid Chromatography (HPLC). Reversed-phase HPLC analysis of reaction mixtures (cf. Table 1) was carried out using an Eurospher 100 C-18 column (250 × 4 mm, 5 *μ*m, Knauer, Berlin, Germany) employing two Knauer HPLC pumps 64. Separation was performed using isocratic conditions (1% acetic acid in water/methanol, 7:3) with a flow rate of 1 mL/min. An HP-1040A

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Table 1. Preparation of Phenyl 6′-*O*-Malonyl-β-D-glucopyranoside: Influence of Reaction Conditions on Product Composition

	reaction conditions					product distribution (area %)		
exp	solvent	vol (mL)	molar ratio **1**:malonic acid:^{t}BuNC	educt **1** (mmol)	reaction time (days)	**1**	**2**	byproducts[a]
1	THF	5.0	1.0:1.9:2.4	0.54	3	70	22	8
2	THF	5.0	1.0:3.9:1.9	0.48	2	10	59	31
3	CH_3CN	1.0	1.0:2.0:2.0	0.039	1	13	45	42
4	CH_3CN	1.0	1.0:1.0:1.5	0.039	1	26	49	25
5	CH_3CN	1.0	1.0:4.5:2.5	0.039	1	<1	17	82
6	CH_3CN	1.0	1.0:2.0:0.8	0.039	1	49	39	12
7	CH_3CN	1.0	1.0:3.0:1.5	0.039	1	13	55	32
8	DMSO	1.0	1.0:3.0:1.5	0.039	1	100		
9	Pyridine	1.0	1.0:3.0:1.5	0.039	1	100		

[a] Tentatively identified as positional isomers and multiple malonylated derivatives of **1** by HPLC−MS/MS and UV detection, respectively.

Table 2. ^{1}H NMR Spectral Data of Compounds 1, 2, and 3[a]

atom	**1**	**2**	**3**
phenyl			
HC-4	7.07 (7.18) t	7.07−7.11	
HC-2, HC-6	7.11 (7.72) d	7.08 (7.36) d	
HC-3, HC-5	7.38 (7.36) t	7.38 (7.38) t	
glucose			
HC-1′	4.93 (7.36) d	4.94 d	
HC-2′, HC-3′, HC-4′, HC-5′	3.22−3.43 m	3.33−3.60 m	
H_aC-6′	3.78 (11.76, 5.34) dd	4.47 (11.40) d	
H_bC-6′	3.54 (11.98, 6.00) dd	4.16 (11.76, 6.96) dd	
malonyl			
H_2C-2″		3.45, 3.44	3.43 s
ethyl			
H_2C-4″			4.17 (7.11) q
$H_{3C-5''}$			1.27 (7.16) t

[a] Recorded in DMSO-d_6, 400 MHz, δ in ppm relative to the signal of DMSO-d_6, coupling constants (J) in hertz. s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

Table 3. ^{13}C NMR Spectral Data of Compounds 1, 2, and 3[a]

assignment	**1**	**2**	**3**	DEPT
phenyl				
C-2, C-6	116.4	116.4		CH
C-4	121.8	121.9		CH
C-3, C-5	129.4	129.4		CH
C-1	157.6	157.4		C
glucose				
C-1′	100.7	100.5		CH
C-2′	73.4	73.3		CH
C-3′	76.8	76.5		CH
C-4′	70.0	70.0		CH
C-5′	77.1	73.8		CH
C-6′	60.9	64.3		CH_2
malonyl				
C-1″		166.8	167.0	C
C-2″		41.6	41.6	CH_2
C-3″		167.8	168.0	C
ethyl				
C-4″			60.6	CH_2
C-5″			14.0	CH_3

[a] Recorded in DMSO-d_6, 400 MHz, δ in ppm relative to the signal of DMSO-d_6.

photodiode array detector (spectral range, 190−600 nm; sampling interval, 640 ms) with an HP-300 workstation was employed at 265 nm. Area percent calculations (cf. Table 1) were based on the integration of peaks occurring at 265 nm.

Purification of Phenyl 6′-*O*-Malonyl-β-D-glucopyranoside (2). Semipreparative reversed-phase HPLC analysis of the reaction mixture obtained in the experiment 1 (cf. Table 1) was carried out using an Eurospher 100 C-18 column (250 × 16 mm, 5 μm, Knauer). Separation was achieved by applying a linear gradient at a flow rate of 5 mL/min. As solvents A and B 1% acetic acid in water and methanol were used, respectively. The gradient program was as follows: 0−15 min, 30% B; 15−20 min, 30−65% B; 20−25 min, 65−80% B; 25−30 min, 80−95% B; 30−35 min, 95% B. UV detection was achieved at 265 nm on a Knauer variable-wavelength UV/Vis detector (3-mm flow cell). Half of the reaction mixture (assay 1; cf. Table 1) was purified, yielding pure **2** as white syrup (21 mg). The ^{1}H NMR and ^{13}C NMR data as well as the LC−MS/MS spectral data are outlined in Tables 2−4, respectively.

Reference Compounds. *(a) Phenyl β-D-Glucopyranoside* ***(1)***. For ^{1}H NMR and ^{13}C NMR data see Table 2 and Table 3. A pseudomolecular ion ($-m/z$ 369 [M + TFA − H]$^-$) was obtained by LC−MS/MS in the negative mode.

(b) Monoethyl Malonate **(3)**. Monoethyl potassium malonate (Hüls AG, Marl, Germany) was converted into **3** by dissolving in 1 N HCl and extraction with diethyl ether. ^{1}H NMR and ^{13}C NMR spectral data are shown in Tables 2 and 3, respectively.

Liquid Chromatography−Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry (LC−APCI-MS/MS). Analysis of synthesized **2** was performed on a triple stage quadrupole TSQ 7000 LC−MC/MS system with atmospheric pressure chemical ionization (APCI) interface (Finnigan MAT, Bremen, Germany) The temperature of the heated vaporizer and inlet capillary in the nebulizer interface were 300 and 180 °C, respectively. The current of the APCI corona discharge needle was set to 5.0 μA, resulting in 4.31-kV needle voltage. Nitrogen served as both sheath (10 L/min) and auxiliary (50 psi) gas. Data acquisition and evaluation were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software (Finnigan MAT, Bremen, Germany). For HPLC, two Knauer HPLC pumps 64 equipped with micropump heads were used. Separations were carried out on an Eurospher 100 C-18 (100 × 2 mm, 5 μm, Knauer) using a linear gradient with a flow rate of 200 μL/min. Solvent A was 0.05% TFA in water; solvent B was 0.05% TFA in methanol. The gradient program was as follows: 0−4 min, 30% B; 4−10 min, 30−50% B; 10−15 min, 50−90% B; 15−17 min, 90% B. Positive and negative modes were applied. Product ion spectra of **2** were available by collision-induced dissociation (CID) (1.8 mTorr argon; ± 15 eV).

Nuclear Magnetic Resonance (NMR). NMR spectra were acquired using a Fourier transform Bruker WM 400 (400 MHz) spectrometer. For the DEPT experiments, the Bruker standard impulse sequence was used. All NMR spectra were recorded in DMSO-d_6 (Aldrich) and referenced to the solvent signal.

RESULTS AND DISCUSSION

In 1977 Rehn and Ugi described the synthesis of malonic acid monoesters under mild conditions using ^{t}BuNC as activating agent for the acid and the esterifying alcohol as solvent. The conversion of ^{t}BuNC into the corresponding N-monosubstituted formamide consumes the water released during esterification, providing the thermodynamic driving force for the overall reaction. We adapted this method for the malonylation

Table 4. Mass Spectral Data of Compound 2 Obtained by HPLC−APCI-MS/MS on RP-18 with a Water/Methanol/TFA Gradient

APCI negative mode[a]		APCI positive mode[a]	
−m/z[b] (200−800)	assignment	+m/z[b] (150−500)	assignment
455	$[M + TFA - H]^-$	205	$[M - phenol - CO_2 + H]^+$
297	$[M - CO_2 - H]^-$	231	$[M - phenol - H_2O + H]^+$
341	$[M - H]^-$	187	$[M - phenol - H_2O - CO_2 + H]^+$
		360	$[M + NH_4]^+$
		316	$[M - CO_2 + NH_4]^+$
	product ions[c] of *m/z* 455		product ions[d] of *m/z* 360
297	$[M - CO_2 - H]^-$	145	$[M - phenol - malonic\ acid + H]^+$
113	$[TFA - H]^-$	127	$[M - phenol - H_2O - malonic\ acid + H]^+$
455	$[M + TFA - H]^-$	231	$[M - phenol - H_2O + H]^+$
203	$[M - phenol - CO_2 - H]^-$	360	$[M + NH_4]^+$
341	$[M - H]^-$	249	$[M - phenol + H]^+$
162	$[C_6H_{10}O_5]^-$	105	$[malonic\ acid + H]^+$

[a] Mass spectra obtained during HPLC analysis of crude reaction mixtures (cf. Table 1) and of purified **2**. [b] Fragments ±*m/z* are ordered by decreasing relative abundance. [c] CID with 1.8 mTorr argon, +15 eV; mass range 20−800 −*m/z*. [d] CID with 1.8 mTorr argon, −15 eV; mass range 20−400 +*m/z*.

of glycosides. **1** served as a model glycoside and was esterified with malonic acid and ^{t}BuNC in aprotic solvents. Our first synthesis was performed in 5 mL of dry THF with 0.54 mmol **1**, 0.95 mmol malonic acid, and 1.20 mmol ^{t}BuNC (cf. Table 1). After 3 days, the solvent was evaporated and the reaction mixture dissolved in methanol. Subsequent RP-18 HPLC analysis with photodiode array detection revealed six peaks (**1** enclosed) with identical UV spectra (maximum at 265 nm, shoulders at 258 and 272 nm). Besides **1** (70% area; 49 mg; 0.19 mmol) eluting at 4.5 min, a second major peak (22% area; 21 mg; 0.06 mmol) was observed at 8.4 min. The second peak was obtained in pure form by semipreparative HPLC on RP-18. In the ^{1}H NMR spectrum (DMSO-d_6, Table 2) of **2**, the two signals at 4.47 (H_aC-6′) and 4.16 ppm (H_bC-6′) were assigned to the C-6′ methylene group of the sugar. Their distinct downfield shift of 0.69 and 0.62 ppm compared with H_aC-6′ and H_bC-6′ in **1**, respectively, suggested that the point of attachment of the malonyl group was C-6′. The two signals at 3.45 and 3.44 ppm in **2** were assigned to the two malonyl methylene protons H_2C-2″, indicating the AB system for these protons (Cheminat et al., 1989), in contrast to the sharp singlet of **3** at 3.43 ppm. The triplet of HC-4 in **1** was overlapped by the strong doublet of HC-2 and HC-6 in **2**. The ^{13}C NMR spectrum of **2** (DMSO-d_6, Table 3) revealed good coincidence with **1**, except for C-5′ and C-6′. The acylation of the C-6′ hydroxyl group in **2** was further confirmed by the chemical shift of C-5′ that appeared 3.33 ppm upfield and that of C-6′ 3.35 ppm downfield from **1**, a characteristical shift pattern in 6-O-acylated glycosides (Yoshimoto et al., 1980). The signals of the malonyl moiety C-1″, C-2″, and C-3″ (166.82, 41.3, and 167.76 ppm) corresponded with those of **3**, clearly indicating the monoester of malonic acid in **2**.

In addition, mass spectral data obtained by LC−APCI-MS/MS analysis (Table 4) were also in accordance with structure **2**. Whereas in the negative scan mode the TFA adduct $[M + TFA - H]^-$ dominated besides the pseudomolecular ion $[M - H]^-$ and the decarboxylation product $[M - CO_2 - H]^-$, the positive scan mode revealed more complex spectral information with $[M - phenol - CO_2 + H]^+$ as major ion. In both cases (negative/positive mode) the aglycon was not detectable. Thus, the ^{1}H NMR and ^{13}C NMR together with LC−APCI-MS/MS mass spectral data revealed the structure of the major reaction product to be **2**.

In order to optimize the reaction conditions of the malonylation, several attempts were made with different molar ratios of educts in various aprotic solvents (Table 1). While DMSO and pyridine were not suitable solvents for the acylation, good yields of up to 60% for **2** were achieved in THF and acetonitrile. From these data we concluded that a 3−4-fold molar excess of malonic acid and an up to 2-fold molar excess of ^{t}BuNC are ideal to achieve good yields of 6-*O*-malonyl glucosides. To the best of our knowledge 60% overall yield for the synthesis of a 6-*O*-malonyl glucoside had not been achieved before (Kasai et al. 1981, Matern et al. 1983). Since Edwards et al. (1986) have given neither experimental details on the synthesis nor spectral data of **2** in their report about its metabolism in rats, we present herewith the first structural elucidation of phenyl 6′-*O*-malonyl-*β*-D-glucopyranoside based on NMR and mass spectral data.

As to the analysis of glycoconjugated flavor precursors, the described one-step reaction opens the way for the facile preparation of reference substances, which are required for the spectroscopic identification of malonylated glycosides in complex natural matrices. The recent identification of 2,5-dimethyl-4-hydroxy-3[2*H*]-furanone 6′-*O*-malonyl-*β*-D-glucopyranoside in strawberry fruit (Roscher et al., 1996) is an impressive example.

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