Cyrmenins, Novel Antifungal Peptides Containing a Nitrogen-Linked β-Methoxyacrylate Pharmacophore: Isolation and Structural Elucidation[‡]

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Dedicated to Professor Wolfgang Steglich on the occasion of his 70th birthday

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The novel antifungal metabolites cyrmenin A, B_1, and B_2 (**1**–**3**) were isolated from *Archangium gephyra* and *Cystobacter armeniaca* strains (myxobacteria). The cyrmenins are modified *N*-acyldipeptide esters containing a didehydroalanine, a 3-*O*-methyl-didehydroserine and a (2*E*,4*Z*)-undecadienoic or -dodecadienoic acid residue. These compounds represent the first bacterial counterparts of strobilurins that are characterized by an α-substituted β-methoxyacrylate pharmacophore.

Introduction

In the course of our screening of myxobacteria for biologically active metabolites, we noticed that strains of *Cystobacter armeniaca* and *Archangium gephyra* had strong antifungal activity. According to HPLC, UV spectroscopy, and MS analyses of crude extracts, the compounds responsible for this activity were not related to the antifungal compounds isolated previously from myxobacteria.[2,3] Here we report the isolation and structural elucidation of the major components from *A. gephyra*, named cyrmenins A (**1**), B_1 (**2**), and B_2 (**3**). In their pure states, the cyrmenins exhibit high activity against, for example, *Botrytis cinerea*, *Phythium debaryanum*, *Hansenula anomala*, *Metschnikowia pulcherrima*, but they are inactive against bacteria.[1b] Like the strobilurin[4] and myxothiazol[5] groups of fungicides, the cyrmenins[1b] are inhibitors of mitochondrial respiration at the cytochrome bc_1 complex. In spite of this general mechanism of action, the cyrmenins exhibit an exceptionally low toxicity for animal cell cultures.[1b]

Results and Discussion

The cyrmenins were recovered by methanol extraction of the amberlite XAD 16 adsorbent resin present during a 300-L fermentation of *A. gephyra*. Evaporation of the methanol from the extract and extraction of the aqueous residue with ethyl acetate gave 80 g of the crude product. This material was partitioned between methanol and heptane to remove the lipophilic components. Further purification and isolation of **1**−**3** was accomplished by three consecutive chromatographic steps on silica gel, RP-18 silica gel, and Sephadex LH-20. Because of the considerable decomposition of the cyrmenins that occurs under basic conditions, all extractive steps and chromatographic separations had to be performed with control of pH (6.5). When these precautions were taken, the pure cyrmenins **1**−**3** were obtained as colorless oils: cyrmenin A (**1**; 440 mg), cyrmenin B_1 (**2**; 492 mg), and cyrmenin B_2 (**3**; 60 mg).

The structural elucidation of the cyrmenins was mainly based on data obtained from cyrmenin B_2 (**3**). High-resolution (+)-DCI MS of the protonated molecular ion at m/z = 393 suggested the elemental composition $C_{21}H_{32}N_2O_5$, which implies the presence of seven double-bond equivalents. The UV spectrum of **3** shows one intense, broad band at 259 nm, while the IR spectrum indicates ester and amide groups by bands at 1720, 1658, and 3360 cm^{-1}. All the signals in the ^1H and ^{13}C NMR spectra were assigned and correlated by $^1H,^1H$-COSY and direct $^1H,^{13}C$-correlation (HMQC) NMR spectroscopy (Table 1). By this means, the constitution of the hydrocarbon fragment, which is marked by bold bonds in Figure 1, was derived straightforwardly. $^1H,^{13}C$ long-range correlations between C-7 and both 8-Me and 6-H completed the 2,10-dimethylundeca-2,4-dienamide partial structure. Its (8*E*) configuration and preferred conformation were verified by the observation of strong NOEs between protons 6-H and 8-Me and protons 8-Me and 10-H. The configuration of the Δ^{10} unit was deduced to be (*Z*) by the coupling constant between protons 10-H and 11-H (10.9 Hz), which is in the range typical for (*Z*) double bonds, and by an NOE between protons 9-H and 12-H.[6]

[‡] Antibiotics from Gliding Bacteria, 96. Part 95: Ref.[1a]

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Table 1. NMR spectroscopic data ($CDCl_3$, 400/100 MHz) for cyrmenin B_2 (**3**)

Position	δ_H	M[a]	J [Hz]	δ_C	M[a]	Position	δ_H	M[a]	J [Hz]	δ_C	M[a]
1	–	–	–	165.2	s	8	–	–	–	129.6	s
1-OMe	3.74	s	–	52.0	q	8-Me	1.99	d	1.5	12.5	q
2	–	–	–	106.7	s	9	7.30	tq	1.3/11.7	129.9	d
2′	7.33	s	–	155.3	d	10	6.24	ddt	1.7/10.9/11.7	123.5	d
2′-OMe	3.90	s	–	62.4	q	11	5.80	dt	7.8/10.9	139.8	d
3	7.18	br. s	–	–	–	12	2.28	dq	1.7/7.4	28.2	t
4	–	–	–	162.8	s	13	1.39	m	–	29.8	t
5	–	–	–	134.2	s	14	1.28	m	–	27.1	t
5′a	6.66	d	1.8	102.4	t	15	1.15	m	–	38.9	t
5′b	5.41	t	1.5	–	–	16	1.49	m	–	28.0	d
6	8.51	br. s	–	–	–	17	0.84	t	6.6	22.6	q
7	–	–	–	167.9	s						

[a] Multiplicity.

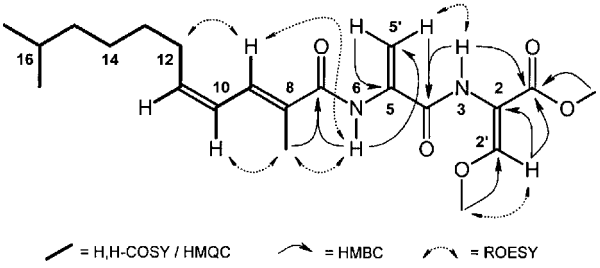
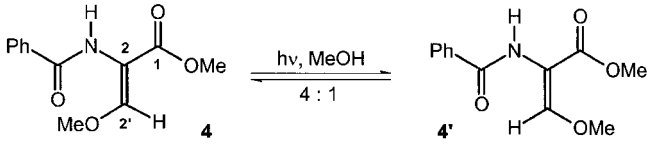


Figure 1. Structural elements of cyrmenin B_2 (**3**) and selected correlations deduced from NMR spectra

Extending this partial structure to include a dipeptide formed from didehydroalanine and 3*O*-methyl-didehydroserine methyl ester was deduced by $^1H,^{13}C$ long-range correlation (HMBC) NMR spectroscopy (Figure 1). Strong long-range interactions between the amide proton 6-H and C-5′ and between C-5 and both protons 5′-H_a and 5′-H_b, as well as the interaction between each of the methylene protons 5′-H and carbonyl C-4, substantiated the presence of the didehydroalanine fragment. Carbonyl atom C-4, on the other hand, is linked to the nitrogen atom of an α-aminoacrylate fragment, as was indicated by long-range correlations between 3-H and each of the carbonyl atoms C-1 and C-4 of the carboxy groups. Similarly, the methoxy group and hydrogen atom in the β-position exhibited the expected C,H correlations with the C-1, C-2 and C-2′ atoms.

Remarkably, only one geometric isomer of the enol ether of cyrmenin was isolated from the fermentation, and attempts to determine its configuration by NOE failed. Under a variety of conditions no NOE was observed between the ester methoxy group and either the 2′-H or 2′-OMe units. Therefore, we synthesized two model compounds, **4** and **4′**, of the corresponding (*E*)/(*Z*) isomers from 4-methoxymethylene-2-phenyl-4*H*-oxazol-5-one.[7] Methanolysis of the oxazolone in the presence of DMAP furnished two products: the desired methyl 2-benzoylamino-3-methoxyacrylate (**4**) and methyl 2-benzoylamino-3,3-dimethoxypropanoate.[8,9] After chromatographic separation, **4** was photoisomerized to yield a 4:1 equilibrium mixture of the (*E*)/(*Z*) isomers of the β-methoxyacrylate, **4** and **4′** (see Scheme 1).



Scheme 1. Photoisomerization of model compound **4**

Stereochemical assignment of the double bond configuration was accomplished by a ROESY NMR spectrum of this mixture.[10] The major isomer **4** displayed strong interactions between the 2′-H proton and the 1-OMe and 2′-OMe units, whereas only one interaction, between 2′-H and 2′-OMe, was observed for the minor isomer **4′**. These features proved the (*Z*) configuration for **4** and the (*E*) configuration for **4′**. Based on these observations, cyrmenin B_2 (**3**) should have the (*E*) configuration because we observed only one NOE interaction between the 2′-H proton and the 2′-OMe group (see above). Inspection of the NMR spectroscopic chemical shifts, however, clearly indicates the opposite assignment (Table 2).

Table 2. Selected NMR spectroscopic chemical shifts of cyrmenin B_2 (**3**) and model compounds **4** and **4′**

	^1H NMR			^{13}C NMR		
	3	**4**	**4′**	**3**	**4**	**4′**
1-OMe	3.74	3.72	3.83	52.0	51.8	52.3
2	–	–	–	106.7	107.5	108.7
2′	7.33	7.31	8.08	155.3	154.8	155.3
2′-OMe	3.90	3.86	3.89	62.4	62.1	62.8

Whereas the ^{13}C NMR spectroscopic chemical shifts are essentially identical for **4**, **4′**, and **3**, and, thus, are not useful for an assignment, the 2′-H chemical shifts are significantly different. In the (*E*) isomer **4′**, the 2′-H proton is strongly deshielded by the C-4 carbonyl group and its signal is shifted to δ = 8.08 ppm. In the (*Z*) isomer **4** and cyrmenin

B_2 (**3**), the 2′-H signals are located at δ = 7.31 and 7.33 ppm, respectively. Therefore, disregarding the lack of an NOE between the 2′-H and 1-OMe units, we assign cyrmenin B_2 (**3**) and its relatives **1** and **2** to have a (2*Z*) configurations.[11] This assignment is in line with the biological activity observed for cyrmenins; during SAR studies of strobilurin derivatives, the (*E*) configuration [corresponding to the (*Z*) configuration in cyrmenins] was found to be essential[4b,12] for biological activity.

From the ^{1}H and ^{13}C NMR spectroscopic data, cyrmenin A (**1**) is characterized as a higher homologue of B_2 (**3**) because it contains an additional methylene group in the fatty acid part. Cyrmenins B_1 (**2**) and B_2 (**3**) are isomers and were separated only with difficulty by chromatography. In cyrmenin B_1 (**2**), the isopropyl terminus of B_2 (**3**) is replaced by an *n*-propyl unit (Figure 2). From a biosynthetic point of view, these modifications arise from different starter units in the polyketide synthesis of the side chain. As in several other myxobacterial metabolites, the starter CoA esters are derived by oxidative degradation of the amino acids leucine, valine, and norvaline.[13]

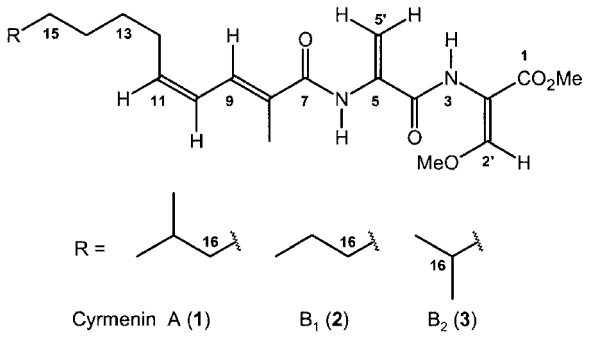


Figure 2. Cyrmenin A (**1**), B_1 (**2**) and B_2 (**3**)

Conclusion

With their two adjacent didehydroamino acids, the cyrmenins represent a unique series of natural products. Although the *O*-methyl-didehydroserine unit is not known to exist in other natural products, its carbon-atom backbone is identical to the well-known β-methoxyacrylate pharmacophore of two related families of natural fungicides. The strobilurins (e.g., A, **5**) and oudemansins[14] were discovered in 1976 by Steglich and Anke as new class of fungicides. They have in common a β-methoxyacrylate unit that is linked to the rest of the molecule by the α-carbon atom, and they are all produced by higher fungi. The second family is produced by myxobacteria and comprises myxothiazoles (e.g., Z, **7**),[15] melithiazoles[16] (= cystothiazols[17]), and haliangicin.[18] They are characterized by the presence of a β-methoxyacrylamide or methyl ester unit, which is linked by the β-carbon atom to the rest of the molecule. Both the α- and β-linked acrylates were identified by Jagow et al.[4c] to be inhibitors of mitochondrial respiration at the cytochrome bc_1 complex. Most remarkably, the cyrmenins are the first α-linked β-methoxyacrylates produced by bacteria that use an entirely different biosynthetic route. Occupying the same binding site on the cytochrome bc_1 complex, the strobilurins, myxothiazoles, and cyrmenins are examples of convergent evolution on the molecular level (see Figure 3).

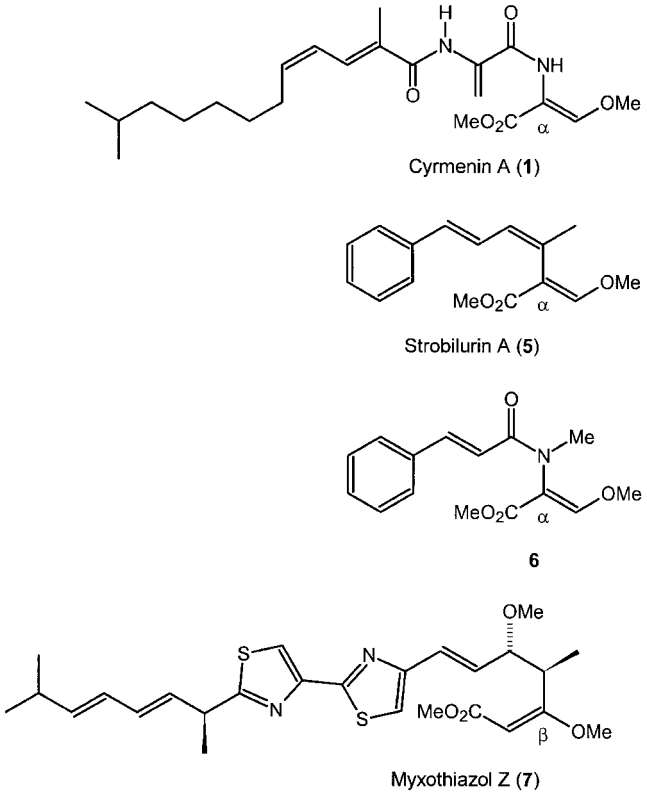


Figure 3. Natural and synthetic β-methoxyacrylates

Because of their relatively simple structure and low mammalian toxicities, the strobilurins have been optimized extensively by chemical synthesis and developed to commercially important agricultural fungicides.[19] Interestingly, during this process, the nitrogen-linked pharmacophore of the cyrmenins has already been anticipated by synthetic chemists (see, e.g., **6**),[20] but it was not developed further.

Experimental Section

General: UV: Shimadzu UV/Vis scanning spectrometer UV-2102, ethanol as solvent [Uvasol, Merck]. IR: Nicolet 20 DXB FTIR spectrometer. NMR: Bruker DMX 600 (^{1}H: 600.1 MHz; ^{13}C: 150.9 MHz), ARX 400 (^{1}H: 400.1 MHz; ^{13}C: 100.6 MHz), AM 300 (^{1}H: 300.1 MHz; ^{13}C: 75.5 MHz) spectrometer; the internal standard was the signal of the solvent. Mass spectrometry (DCI): Finnigan MAT 95 spectrometer (DCI with isobutane), resolution M/ΔM = 1000; high-resolution data from peak matching (M/ΔM = 10000).

Isolation of Cyrmenins: The adsorber resin was harvested by centrifugation of the fermentation batch (300 L) of the *Archangium gephyra* strain Ar 9944 grown in the presence of Amberlite XAD 16 (2 L). The collected cell mass and adsorber resin (7.48 kg) were

extracted with methanol (7 × 2.5 L, total 17.5 L). The aqueous phase (2.25 L) that remained after evaporation of the organic solvent, was adjusted to pH = 7 and extracted four times with ethyl acetate (5 L in all). The organic layer was dried with magnesium sulfate and the solvents were evaporated. To remove the lipophilic components, the residue (80 g) was partitioned between methanol containing 3% water (800 mL) and heptane (3 × 800 mL). Concentration of the polar layer yielded the raw product (53 g), which was separated by silica gel chromatography [LiChroprep Si 100 silica gel (540 g), 63−200 μm (Merck), 9 × 60 cm; eluents: CH_2Cl_2 (4 L) and CH_2Cl_2/diethyl ether (95:5; 6 L)]. After HPLC analysis, the fractions containing cyrmenins (**1**−**3**) were combined and concentrated to dryness (residue 2.55 g). LH-20 chromatography with dichloromethane/methanol (4:1) as solvent removed the major part of the impurities to yield the cyrmenin complex (1.40 g). Finally, RP-MPLC separation [3 portions, HD-Sil, 60 Å, 20 μm (YMC); 6 × 50 cm; eluent: CH_3CN/50 mM NH_4OAc buffer; detection: UV absorption at 277 nm] of this mixture furnished cyrmenin A [**1**; 440 mg (corresponding to 1.47 mg/mL of fermentation broth)], cyrmenin B_1 [**2**; 492 mg (corresponding to 1.64 mg/mL of fermentation broth)], and cyrmenin B_2 [**3**; 60 mg (corresponding to 0.20 mg/mL of fermentation broth)] as colorless oils.

Cyrmenin A (1): UV (ethanol): $\lambda_{max.}$ (lg ε) = 256 (4.28) nm. IR (KBr): $\tilde{\nu}$ = 3377 (w), 2953 (m), 2926 (m), 2854 (w), 1720 (m), 1658 (s), 1496 (s) cm^{-1}. ^1H NMR (400.1 MHz, $CDCl_3$): δ = 0.85 (d, *J* = 6.6 Hz, 6 H, 18-H_3/19-H_3), 1.14 (m, 2 H, 16-H), 1.27 (m, 4 H, 14-H/15-H), 1.40 (m, 2 H, 13-H), 1.50 (m, *J* = 6.6 Hz, 1 H, 17-H), 1.99 (s, 3 H, 8-CH_3), 2.28 (m, *J* = 1.6, 7.2 Hz, 2 H, 12-H), 3.76 (s, 3 H, 1-OCH_3), 3.91 (s, 3 H, 2′-OCH_3), 5.39 (br. s, 1 H, 5′-H_b), 5.80 (dt, *J* = 7.2, 10.7 Hz, 1 H, 11-H), 6.24 (m, *J* = 1.6, 10.7, 11.8 Hz, 1 H, 10-H), 6.67 (br. s, 1 H, 5′-H_a), 7.08 (br. s, 1 H, 3-H), 7.30 (br. d, *J* = 11.7 Hz, 1 H, 9-H), 7.33 (s, 1 H, 2′-H), 8.50 (br. s, 1 H, 6-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 167.7 (s, C-7), 165.0 (s, C-1), 162.6 (s, C-4), 155.0 (d, C-2′), 139.8 (d, C-11), 134.2 (s, C-5), 129.8 (d, C-9), 129.5 (d, C-8), 123.4 (d, C-10), 106.7 (s, C-2), 102.2 (t, C-5′), 62.4 (q, 2′-OCH_3), 52.0 (q, 1-OCH_3), 38.9 (t, C-16), 29.5 (2 t, C-13 / C-14), 28.2 (t, C-12), 27.9 (d, C-17), 27.3 (t, C-15), 22.6 (q, C-18), 12.5 (q, 8-CH_3) ppm. DCI MS (+, NH_3): *m*/*z* (%) = 424 (100) $[M + NH_4]^+$. HR-DCI MS: $C_{22}H_{38}N_3O_5$: calcd. 424.2811 $[M + NH_4]^+$; found 424.2821. t_R = 6.29 min (CH_3CN/5 mM NH_4OAc buffer, 63:37).

Cyrmenin B_1 (2): UV (ethanol): $\lambda_{max.}$ (lg ε) = 258.0 (4.22) nm. IR (KBr): $\tilde{\nu}$ = 3380 (w), 2927 (m), 2854 (w), 1719 (m), 1658 (s), 1496 (s) cm^{-1}. ^1H NMR (400.1 MHz, $CDCl_3$): δ = 0.87 (t, *J* = 7.1 Hz, 3 H, 18-H), 1.25 (m, 8 H, 14-H/15-H/16-H/17-H), 1.37 (m, 2 H, 13-H), 1.99 (d, *J* = 1.4 Hz, 3 H, 8-CH_3), 2.28 (dq, *J* = 1.7, 7.4 Hz, 2 H, 12-H), 3.76 (s, 3 H, 1-OCH_3), 3.91 (s, 3 H, 2′-OCH_3), 5.39 (t, *J* = 1.6 Hz, 1 H, 5′-H_b), 5.80 (m, *J* = 0.8, 7.6, 11.3 Hz, 1 H, 11-H), 6.24 (m, *J* = 1.7, 11.1, 11.4 Hz, 1 H, 10-H), 6.66 (d, *J* = 1.7 Hz, 1 H, 5′-H_a), 7.08 (br. s, 1 H, 3-H), 7.30 (m, *J* = 1.4, 1.5, 11.8 Hz, 1 H, 9-H), 7.32 (s, 1 H, 2′-H), 8.49 (br. s, 1 H, 6-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 167.8 (s, C-7), 165.2 (s, C-1), 162.8 (s, C-4), 155.4 (d, C-2′), 139.8 (d, C-11), 134.1 (s, C-5), 129.8 (d, C-9), 129.6 (d, C-8), 123.4 (d, C-10), 106.7 (s, C-2), 102.4 (t, C-5′), 62.3 (q, 2′-OCH_3), 51.9 (q, 1-OCH_3), 31.8 (t, C-16), 29.5 (t, C-13), 29.2 (t, C-14), 29.1 (t, C-15), 28.1 (t, C-12), 22.6 (t, C-17), 14.0 (q, C-18), 12.4 (q, 8-CH_3) ppm. DCI MS (+, NH_3): *m*/*z* (%) = 393 (1) $[M + H]^+$, 410 (100) $[M + NH_4]^+$. HR-DCI MS: $C_{21}H_{36}N_3O_5$ $[M + NH_4]^+$: calcd. 410.2655; found 410.2701. t_R = 4.74 min (CH_3CN/5 mM NH_4OAc buffer, 63:37).

Cyrmenin B_2 (3): UV (ethanol): $\lambda_{max.}$ (lg ε) = 257.5 (4.21) nm. IR (KBr): $\tilde{\nu}$ = 3377 (w), 2953 (m), 2927 (m), 2854 (w), 1720 (m), 1658 (s), 1496 (s) cm^{-1}. DCI MS (+, NH_3): *m*/*z* (%) = 410 (100) $[M + NH_4]^+$. HR-DCI MS: $C_{21}H_{36}N_3O_5$ $[M + NH_4]^+$: calcd. 410.2655; found 410.2678. t_R = 4.32 min (CH_3CN/5 mM NH_4OAc buffer, 63:37).

Synthesis of Reference Compounds

Synthesis of Methyl (*Z*)-2-Benzoylamino-3-methoxyacrylate (4) and Methyl 2-Benzoylamino-3,3-dimethoxypropanoate: A solution of 4-methoxymethylene-2-phenyl-4*H*-oxazol-5-one[7a] (50 mg, 0.25 mmol) and 4-DMAP (10 mg, 82 μmol, 0.33 equiv.) in MeOH (abs.; 2.5 mL) was stirred at room temp. for 2 h. The solvent was evaporated in vacuo and the resulting oil was purified by chromatography (silica gel; CH_2Cl_2/Et_2O, 9:1) to yield **4** as colorless crystals (8.6 mg, 37 μmol, 15%) and the acetal as a colorless solid (51 mg, 0.19 mmol, 76%).

4: M.p. 152−154 °C (benzene) [ref.[21] 155−156 °C (benzene)]. ^1H NMR ($CDCl_3$, 300.1 MHz): δ = 3.72 (s, 3 H, 1′′-H), 3.86 (s, 3 H, 4-H), 7.17 (br. s, 1 H, NH), 7.31 (s, 1 H, 3-H), 7.41 (m, 2-H, 4′-H), 7.48 (m, 1-H, 5′-H), 7.83 (m, 2 H, 3′-H) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 51.8 (q, C-1′′), 62.1 (q, C-4), 107.5 (s, C-2), 127.5/128.5 (2 d, C-3′/C-4′), 131.7 (d, C-5′), 134.0 (s, C-2′), 154.8 (d, C-3), 165.5 (s, C-1′), 165.6 (s, C-1) ppm.

Methyl 2-Benzoylamino-3,3-dimethoxypropanoate: ^1H NMR ($CDCl_3$, 400 MHz): δ = 3.43 (s, 6 H, 4-H), 3.76 (s, 3 H, 1′′-H), 4.68 (d, *J* = 3.4 Hz, 1 H, 3-H), 5.03 (dd, *J* = 3.3, 8.4 Hz, 1 H, 2-H), 6.85 (br. d, *J* = 8.2 Hz, 1 H, NH), 7.44 (m, 3 H, 4′-H, 5′-H), 7.80 (m, 2 H, 3′-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 52.5 (q, C-1′′), 54.4 (d, C-2), 55.7 (q, C-4), 103.8 (d, C-3), 127.2 (d, C-3′), 128.5 /131.8 (2 d, C-4′/C-5′), 133.7 (s, C-2′), 167.5 (s, C-1′), 169.7 (s, C-1) ppm.

Photoisomerization of Methyl (*Z*)-2-Benzoylamino-3-methoxyacrylate (4): A solution of methyl (*Z*)-2-benzoylamino-3-methoxyacrylate (**4**) (20 mg, 85 μmol) and benzophenone (2.0 mg, 11 μmol) in degassed MeOH (abs.; 2.0 mL) was irradiated in a quartz vial for 3 × 30 min at room temp. using a high-pressure mercury vapor lamp (Philips HPK, 125 W). After the equilibrium was reached, at a (*Z*)/(*E*) ratio of ca. 4:1, the solvent was evaporated in vacuo and the resulting oil was purified by preparative HPLC (VP, 250/21 Nucleodur 100−10 C18 EC; CH_3CN/50 mM NH_4OAc buffer, 1:9). A mixture of (*Z*) isomer **4** and (*E*) isomer **4′** (19 mg, 93%) was isolated.

4′: ^1H NMR ($CDCl_3$, 300.1 MHz): δ = 3.83 (s, 3 H, 1′′-H), 3.89 (s, 3 H, 4-H), 7.41 (m, 2-H, 4′-H), 7.48 (m, 1-H, 5′-H), 7.79 (m, 2 H, 3′-H), 8.08 (s, 1 H, 3-H) ppm. ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ = 52.3 (q, C-1′′), 62.8 (q, C-4), 108.7 (s, C-2), 126.9/128.7 (2 d, C-3′/C-4′), 131.6 (d, C-5′), 134.4 (s, C-2′), 155.3 (d, C-3), 164.6 (s, C-1′), 165.6 (s, C-1) ppm.

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