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# Intermediates in the Synthesis of Dipyrazolic Podands and Ester Crowns *via* Regioselective Lipase Catalyzed Hydrolysis of a Tetraester

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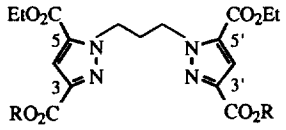
***Abstract.***— In this work we study the *Mucor miehei* lipase-catalyzed hidrolysis of 1,3-bis[3,5-bis(ethoxycarbonyl)-1*H*-pyrazol-1-yl]propane and the potential usefulness of the resulting acids as intermediates in the synthesis of podand and crown esters.


## INTRODUCTION

Crown ethers form a family of compounds that display interesting features derived from their cation complexing properties. Their binding selectivity may be modified by the presence of $sp^2$ nitrogen atoms as a part of the ring and, as a result of this, many crowns and podands (pseudocyclic crown ethers) including nitrogen-containing heterocyclic systems have been synthesized and their properties studied.[1] Our group has published several papers concerning podand and crown molecules containing 1-methyl and 1*H*-substituted pyrazoles.[2] In more recent years, our interest has been focused on the 1,3-bis(1*H*-pyrazol-1-yl)propane unit: podands[3] and crowns[4,5] have been synthesized *via* regioselective transesterification catalyzed by lipases. This is a direct method in one step under easy and mild experimental conditions, but presents the disadvantage that yields, although favourable when compared with classical organic synthesis, are not very high, specially for crowns. An alternative chemoenzymatic route could be to obtain the intermediate diacid **1** by enzymatic hydrolysis to be transformed into the acyl chlorides and then into esters, amides,... by common chemical reactions. It is a more versatile route to many classes of derivatives but introduces two chemical additional steps. Moreover, aromatic and heteroaromatic esters are poor substrates in lipase-catalyzed reactions and there are very few references dealing with their use in transesterification[6] or hydrolytic[7] reactions and frequently describe unsatisfactory results.[8] In this work, we study the enzymatic hydrolysis of 1,3-bis[3,5-bis(ethoxycarbonyl)-1*H*-pyrazol-1-yl]propane **2** and identify the resulting products.




**1**: R = H
**2**: R = Et

## RESULTS AND DISCUSSION

*ENZYMATIC REACTIONS*

The study was carried out using small volume (sealed 2 mL vials) reactions, followed by preparative scale to isolate and identify the products. The main analytical methods used were TLC and HPLC chromatography.

*Enzymes*

Several common lipases (from *Mucor miehei, Candida antarctica* and *rugosa, Pseudomonas sp.* and *fluorescens, Chromobacterium viscosum,* porcine pancreatic, *Penicillium rocheforti, Rizhopus arrhizus, Aspergillus niger* and *Geotrichum candidum,* all of them from usual suppliers) were checked in conditions of hydrolysis, stirring in an orbital shaker 20 mg/mL of the lipase suspended in a 20 mM solution of **2** in buffer saturated diisopropylether at 30°C. The reactions were analyzed by TLC after 24 hours. As expected after our previous experience, only *Mucor miehei* lipase[9] (MML) and, in a much lesser extension, *Candida rugosa* catalyzed the reaction.

*Solvents and extracting method*

It is generally accepted that solvent is a relevant factor to enzymatic efficiency. It may affect the activity[10] of the enzyme and even the regio[11] and enantioselectivity[12] of the reaction although the characteristics of the solvent that determine these catalytic features still remain unclear.[13]

We performed a general survey of solvents: 13.9 mg of **2** were dissolved in the corresponding organic solvent and the solutions were completed with buffer to a final volume of 1.5 mL (20 mM) of the following solvent-buffer media: dioxane 90 and 10%, acetonitrile 90 and 10%, acetone 10%, *terc*-butanol 90 and 10%. Buffer-saturated diisopropylether and toluene were also checked. 30 mg of MML were added to the reactions and the vials were incubated for 24 hours at 60°C in an orbital shaker. Samples of 20 µL were withdrawn, evaporated to dryness, diluted with 200 µL of MeOH and analyzed by HPLC. The highest conversion was obtained in diisopropylether (44%) followed by toluene (20%) and small amounts in the rest. Four main products (**3-6**) were detected, besides the starting substrate **2** (Table 1, method A).

Because of the chelating properties of the propylendipyrazolic derivatives and the probable presence of one or two carboxylic acid groups in the molecule, it can be expected that some material could remain attached to the anion exchange resin used as support of the lipase or to the very enzyme. The reaction was repeated under the same experimental conditions but, after 24 hours, the mixture was evaporated to dryness, extracted three times with boiling methanol and analyzed by HPLC (Table 1, method B):

TABLE 1.— Reaction in buffer-saturated diisopropylether after 24 h. HPLC data.

| | **2** | **3** | **4** | **5 (1)** | **6** |
|---|---|---|---|---|---|
| % (method A) | 56 | 30 | 3 | 6 | 1 |
| % (method B) | 46 | 25 | 3 | 22 | 4 |
| Retention time (min) | 28.7 | 22.8 | 22.2 | 12.8 | 11.8 |

After their retention times we thought that **3** and **4** were monoacids whilst **5** and **6** were diacids. We later confirmed (see *Structural Elucidation* part) that **3** was the 3-monoacid, **4** the 5-monoacid, **5** was the 3,3'-diacid **1** and, finally, **6** was identified as the 3,5'-diacid (Figure 1).

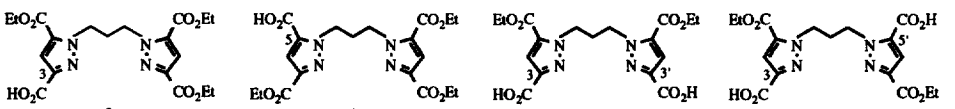



FIGURE 1.— Mono and diacids from the hydrolysis reaction

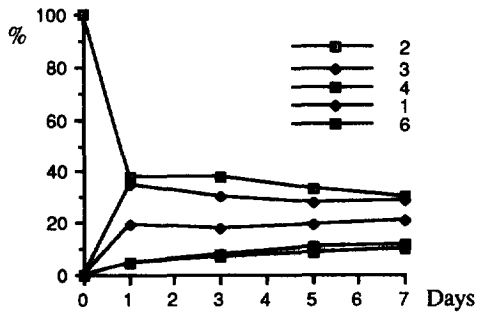
According to the data shown in Table 1, there are two remarkable points. First, the hydrolysis is also a regioselective reaction and ester groups on 3 are preferentially hydrolyzed over those on 5 positions of the ring. Second, both **1** and **6** diacids remained adsorbed on the solid phase (supported enzyme) although they can be extracted when using strong conditions. After this extraction, they both increase their percentage in the mixture by approximately four times.

*Substrate concentrations*

Preparative reactions at a 20 mM concentration are not convenient because of the great volumes of solvent needed. Reactions at 20, 50 and 100 mM concentrations catalyzed by 20, 50 and 100 mg/mL in the same experimental conditions were compared. The kinetic course of the reactions was studied following the above described extractive method B: instead of withdrawing aliquots from a unique reaction, four vials of each concentration were incubated and then processed and extracted after 1, 3, 5 and 7 days. The shape of the curves was nearly identical for the three concentrations. Table and Figure 2 display the data and kinetics of 100 mM reaction.

TABLE and FIGURE 2.— **2** (100 mM) and MML (100 mg/mL) in buffer-saturated diisopropylether, 60°C. HPLC data, method B.

| Days | **2** | **3** | **4** | **1** | **6** |
|---|---|---|---|---|---|
| 1 | 38 | 35 | 5 | 19 | 5 |
| 3 | 38 | 30 | 7 | 18 | 8 |
| 5 | 33 | 28 | 9 | 19 | 11 |
| 7 | 30 | 29 | 10 | 21 | 12 |




*Enzyme inactivation*

Figure 2 shows clearly that the reaction takes place mostly in the first 24 hours and then nearly stops. There are two possibilities: first, it has reached the equilibrium and second, the enzyme has become inactivated.

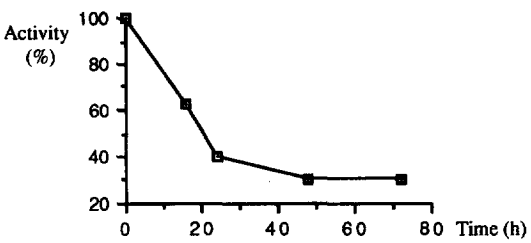
Considering the first alternative, saturated diisopropylether contains 0.4% of water (measured by a Karl Fischer apparatus) dissolved, and the commercial enzyme used is delivered with approximately 10% w/w of water. It means a total water of 14 mg/mL, that is, a concentration 0.78 M which is a reasonable excess over the 100 mM (x 2 hydrolysing ester groups) concentration of **2**. More water was added to the system: first, adding directly 2% of buffer to the reaction mixture and second, increasing the water carried by the enzyme up to roughly 30%,[14] but no conversion was detected in either case. A possible explanation of this lack of reactivity is related to the solubility of **2** in water. The macroporous anion exchange resin which supports the enzyme is highly hydrophilic and tends to be covered by a water layer, draining water from the surrounding liquid or not releasing its original layer. This layer would avoid the lipophilic substrate **2** to reach the enzyme active site. There has recently been published an interesting work[15] dealing with the hydrophilic character of Lipozyme as a whole, its capability to remove water from the organic medium and its influence in the enzyme catalysis.

The second and more convincing alternative is that the enzyme lost its activity in the course of the reaction because of a product inhibition as they are adsorbed on the enzyme when formed. The amount of diacids extracted was significantly increased when the enzyme was treated with boiling methanol (above-mentioned method B). The activity of the enzyme can be easily and quickly measured with a standard reaction, transesterification of tributyrin with a long chain alcohol in an apolar solvent and analyze the formation of the corresponding butyrate by gas chromatography (see *Experimental* part). Comparison of a used enzyme with the original one (assigned activity 100%) affords the remaining activity.

Four reactions were carried out, the solid phase filtered off at different times and the residual activity checked (Table and Figure 3). The enzyme is strongly inhibited during the first day, remaining at a more or less 30% of its original activity after 2-3 days. These results are in accordance with those displayed at Figure 2. We cannot know if the inhibition is reversible and activity recovered after removing the products because the very extractive method inactivates the enzyme: activity disappeared completely and irreversibly when fresh enzyme was suspended after a few minutes in boiling methanol.

TABLE and FIGURE 3.— Remaining activity of MML when used in the reaction

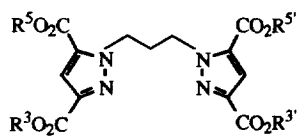
| Time (h) | Activity (%) |
|---|---|
| 0 | 100 |
| 16 | 63 |
| 24 | 40 |
| 48 | 30 |
| 72 | 30 |




*Preparative scale*

In order to isolate and identify the products, hydrolysis of **2** was carried out at a preparative 464 mg, 100 mM scale in buffer-saturated diisopropylether. After stirring at 60°C for 6 days, the solid phase was filtered off, extracted in a Soxhlet apparatus with methanol and the resulting solution mixed with the original mother liquor and chromatographed on a silica gel column. Initially, the yield of separate acids was: 3-monoacid **3** 16%, 5-monoacid **4** 6%, 3,3'-diacid **1** 15% and 3,5'-diacid **6** 9%, but it was increased by eluting the column with methanol to extract the material which remained adsorbed on the silica gel

Derivatives such as esters should be much easier to separate than these acids, whose only importance is as synthetic intermediates. We checked the alternative of synthesizing these derivatives in a "one pot" reaction from the mixture of acids. It was treated with $SOCl_2$ and the resulting mixture of acyl chlorides reacted with diethyleneglycol monomethylether (HO-DEG-OMe) and triethylamine to obtain the mixture of esters **7-10** (Figure 4) that were easily separated, purified and identified. Our group has experience in the chemical esterification of pyrazolic derivatives and, moreover, we have samples of podands **7-9** previously synthesized by us.[3]




**2, 7-10**

| | Yields (%) |
|---|---|
| **2**: $R^3=R^5=R^{3'}=R^{5'}=Et$ | 20 |
| **7**: $R^5=R^{3'}=R^{5'}=Et$, $R^3$=DEG-OMe | 17 |
| **8**: $R^3=R^{3'}=R^{5'}=Et$, $R^5$=DEG-OMe | 5 |
| **9**: $R^5=R^{5'}=Et$, $R^3=R^{3'}$=DEG-OMe | 14 |
| **10**: $R^5=R^{3'}=Et$, $R^3=R^{5'}$=DEG-OMe | 7 |

FIGURE 4.— Isolated podands. Final yields based in the starting tetraester **2**.

*STRUCTURAL ELUCIDATION*

The structures of the new compounds have been established from their spectroscopic data, taking into account that in the $^1H$ NMR spectra of N-alkylated pyrazoles the 3-alkoxy groups are always more deshielded than their counterparts of the 5 position, and in $^{13}C$ NMR the order for pyrazolic carbons is: C(3)>C(5)>C(4) [δ, ppm].[16]

When the $^{13}C$ NMR spectra of monoacids (**3, 4**) and diacids (**1, 6**) were compared to this of the initial tetraethylester (table 4), it was observed a downfield shift of the pyrazolic carbon bound to the carboxylic group, while no appreciable changes were observed in the rest of the molecule.

TABLE 4.— Significant $^{13}C$ NMR data of mono- (**3, 4**) and diacids (**1, 6**) derived from 1,3-bis[3,5-bis(ethoxycarbonyl)-1*H*-pyrazol-1-yl]propane (**2**).

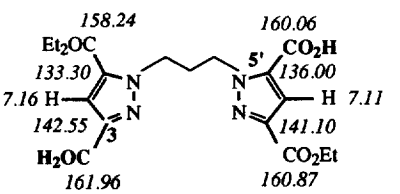
| Compd. | (3)$CO_2H$ | (3)$CO_2Et$ | (5)$CO_2H$ | (5)$CO_2Et$ | (3)*C*-$CO_2H$ | (3)*C*-$CO_2Et$ | (5)*C*-$CO_2H$ | (5)*C*-$CO_2Et$ |
|---|---|---|---|---|---|---|---|---|
| **2**[a] | — | 162.83 | — | 160.08 | — | 143.30 | — | 135.24 |
| **3**[a] | 164.44 | 162.92 | — | 160.19 | 143.89 | 143.34 | — | 135.31 |
| **4**[a] | — | 163.08 | 161.53 | 160.14 | — | 143.37 | 136.25 | 135.36 |
| **2**[b] | — | 160.60 | — | 158.22 | — | 141.44 | — | 133.45 |
| **1**[b] | 162.00 | — | — | 158.34 | 142.48 | — | — | 133.44 |
| **6**[b] | 161.96 | 160.87 | 160.06 | 158.24 | 142.55 | 141.10 | 136.00 | 133.30 |

Solvents: [a]$CD_3OD$. [b]$(CD_3)_2SO$.

However, the diacid **6** showed both $^1H$ and $^{13}C$ resonances in accordance with two posible structures: (i) diacid-3,5 with the two acid groups attached in the same pyrazolic ring, or (ii) diacid-3,5' with the two acid groups in diferent pyrazoles. Its structure (diacid-3,5') was unequivocally demonstrated by a HMBC (Heteronuclear Multiple Bond Correlation) experiment, in which we observed that each aromatic proton correlated with a carboxylic acid and with an ester group (compare tables 4 and 5). This result is only compatible with the structure depicted in figure 5.

TABLE and FIGURE 5.— Correlation proton-carbon (δ, ppm) of diacid **6** by a HMBC experiment and its unequivocal structure

| Aromatic protons | Connected carbons |
|---|---|
| 7.16 | 161.96; 158.24; 142.55; 133.30 |
| 7.11 | 160.87; 160.06; 141.10; 136.00 |




The podand **10**, described here for the first time, showed microanalytical and spectroscopical data in accordance with the proposed structure, with the polyethylene chains situated in the positions 3 and 5',

belonging to different pyrazoles. This structure was also demonstrated by an individual chemical synthesis from its starting diacid **6**, previously isolated among the rest of the acids.

## CONCLUSIONS

3-Mono- and 3,3'-diacids derived from the 1,1'-trimethylenedipyrazole system can be regioselectively obtained by enzymatic hydrolysis of the tetraester **2**. They are useful intermediates to synthesize derivatives such as podand and crown esters *via* classical chemical transformation of the acids into acyl chlorides which are readily esterified in a "one pot" reaction. Unlike the mixture of acids, the resulting esters can be easily purified.

## EXPERIMENTAL

Analytical HPLC was performed on a Beckman chromatograph using a Waters Delta Pak C-18 column (3.9 x 150 mm), eluted with different proportions of acetonitrile and a 0.05% trifluorocetic acid aqueous solution at a flow rate of 1 mL/min and UV detector at λ:233 nm. GC Hewlett-Packard chromatograph was equipped with a 25 m capillary column of phenylmethyl silicone. Analytical TLC was performed on aluminium sheets coated with 0.2 mm layer of silica gel 60 $F_{254}$ (Merck). Chromatographic separations were carried out on columns, using the flash chromatography technique on silica gel (Merck, 230-400 mesh). NMR spectra were recorded using a Varian Unity-500, a Varian XL-300 or a Gemini-200 spectrometer. Buffer phosphate 0.15 M pH: 7.0 was used in all cases. Buffer-saturated diisopropylether was obtained by stirring overnight distilled diisopropylether with buffer at room temperature.

*RESIDUAL ACTIVITY OF USED LIPOZYME*

Fresh and used Lipozyme (10 mg) were added to two vials containing 0.5 mL of a solution of tributyrin (100 mM) and n-octanol (100 mM) in isooctane each. The reactions were incubated at 60°C during 30 minutes and then analyzed by gas chromatography to compare the formation of octyl butyrate catalyzed by the fresh enzyme (given value 100%) and the used one.

*ENZYMATIC HYDROLYSIS AT PREPARATIVE SCALE*

Lipozyme (1 g) was added to a 100 mM solution of **2** (464 mg, 1 mmol) in buffer-saturated diisopropylether (10 mL). The mixture was sealed and incubated at 60°C during 5 days. Afterwards, the solid phase was filtered off and extracted with methanol (150 mL) during 3-4 hours in a Soxhlet apparatus. Diisopropylether mother liquors and methanolic solution were mixed and evaporated to dryness and the residue was chromatographed on a silica gel column, using $CH_2Cl_2$:MeOH:AcOH 14:1:0.04 v/v as eluent.

Initial tetraester **2** (135 mg, 29%) was the first product eluted.

The second fraction (123 mg) was a mixture of the two monoacids **3** and **4**.

The third fraction (38 mg, 9%) consisted of 1,1'-trimethylendipyrazol-3',5-bis(ethoxycarbonyl)-3,5'-dicarboxylic acid **6**, isolated as a colorless powder. $^1$H NMR [$(CD_3)_2SO$]: 7.16 (s, 1H, $H_4$), 7.11 (s, 1H, $H_{4'}$), 4.64 (t, 4H, J=6.8 Hz, $NCH_2$), 4.29 (q, 2H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3'}$), 4.26 (q, 2H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_5$), 2.39 (quint, 2H, J=6.8 Hz, $NCH_2CH_2CH_2N$), 1.29 (t, 3H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3'}$), 1.28 (t, 3H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_5$).

Finally, a fourth fraction (62 mg, 15%) was identified as 1,1'-trimethylendipyrazol-5,5'-bis(ethoxycarbonyl)-3,3'-dicarboxylic acid **1**, isolated as a yellow powder. $^1$H NMR [$(CD_3)_2SO$]: 7.16 (s, 2H, $H_{4,4'}$), 4.58 (t, 4H, J=7.0 Hz, $NCH_2$), 4.27 (q, 4H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{5,5'}$), 2.42 (quint, 2H, J=7.0 Hz, $NCH_2CH_2CH_2N$), 1.29 (t, 6H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{5,5'}$).

A second elution was carried out with the second fraction (123 mg) and two new portions were separated 1,1'-trimethylendipyrazol-3,3',5'-tris(ethoxycarbonyl)-5-carboxylic acid **4** (26 mg, 6%) as a yellow syrup and 1,1'-trimethylendipyrazol-5,3',5'-tris(ethoxycarbonyl)-3-carboxylic acid **3** (70 mg, 16%) as a reddish syrup.

**3:** $^1$H NMR ($CD_3OD$): 7.44 (s, 1H, $H_4$), 7.43 (s, 1H, $H_{4'}$), 4.87 (t, 4H, J=6.8 Hz, $NCH_2$), 4.55 (q, 2H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3'}$), 4.53 (q, 4H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{5,5'}$), 2.71 (quint, 2H, J=6.8 Hz, $NCH_2CH_2CH_2N$), 1.58 (t, 3H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3'}$), 1.55 (t, 6H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{5,5'}$).

**4:** $^1$H NMR ($CD_3OD$): 7.43 (s, 1H, $H_4$), 7.42 (s, 1H, $H_{4'}$), 4.87 (t, 4H, J=6.8 Hz, $NCH_2$), 4.55 (q, 4H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3,3'}$), 4.53 (q, 2H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_5$), 2.69 (quint, 2H, J=6.8 Hz, $NCH_2CH_2CH_2N$), 1.56 (t, 6H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3,3'}$), 1.55 (t, 3H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{5'}$).

*ESTERIFICATION WITH DIETHYLENE GLYCOL MONOMETHYL ETHER (HO-DEG-OMe).*

Tetraester **2** (464 mg, 1 mmol) was hydrolyzed following the procedure described above. The syrupous crude mixture of products (395 mg) was not purified but refluxed in thionyl chloride (2 mL) for 2-3 hours and then evaporated to dryness. The resulting solid was re-dissolved in toluene (5 mL) and treated with a solution (5 mL) 20% v/v of HO-DEG-OMe in thiethylamine. The mixture was stirred and heated at 50°C for 3 hours, processed in a rotary evaporator to remove the excess of amine, diluted with toluene (50 mL), and washed with water (5 x 50 mL). The organic phase was dried ($Na_2SO_4$), the solvent evaporated and the residue eluted with hexane:chloroform:acetone 10:8:1 v/v. The order of elution was **2** (93 mg, 20%), **7** (92 mg, 17%), **8** (27 mg, 5%), **9** (86 mg, 14%) and **10** (43 mg, 7%). All the DEG-OMe esters **7-10** were obtained as colorless syrups. HPLC and NMR data of compounds **7-9** were identical to that of the compounds already prepared and described by us.[3]

1-[3-(3,6-dioxaheptyloxycarbonyl)-5-ethoxycarbonyl-1*H*-pyrazol-1-yl]-3-[3-ethoxycarbonyl-5-(3,6-dioxaheptyloxycarbonyl)-1*H*-pyrazol-1-yl]propane (**10**) was also obtained in 64 % yield from its isolated starting diacid **6**, following the above experimental procedure. $^1$H NMR ($CDCl_3$): 7.33 (s, 1H, $H_4$), 7.30 (s, 1H, $H_{4'}$), 4.66 (t, 4H, J=7.1 Hz, $NCH_2$), 4.46 (t, 2H, J=5.0 Hz, α), 4.42 (t, 2H, J=5.0 Hz, α'), 4.36 (q, 2H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3'}$), 4.29 (q, 2H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_5$), 3.78 (t, 2H, J=5 Hz, β), 3.76 (t, 2H, 4.9 Hz, β'), 3.63 (m, 4H, γ,γ'), 3.52 (m, 4H, δ,δ'), 3.34 (s, 6H, $OCH_3$), 2.44 (quint, 2H, J=7.1 Hz, $NCH_2CH_2CH_2N$), 1.35 (t, 3H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_{3'}$), 1.33 (t, 3H, J=7.1 Hz, $CH_3CH_2CO_2$-$C_5$). $^{13}$C NMR ($CDCl_3$): 161.50 and 161.40 (O=*C*-$C_{3,3'}$), 158.95 and 158.86 (O=*C*-$C_{5,5'}$), 142.46 and 142.00 ($C_{3,3'}$), 133.62 and 133.33 ($C_{5,5'}$), 114.46 and 114.28 ($C_{4,4'}$), 71.94 (δ,δ'), 70.64 (γ'), 70.56 (γ), 69.06 (β), 68.88 (β'), 64.43 (α'), 64.03 (α), 61.46 ($CH_3CH_2CO_2$-$C_5$), 61.20 ($CH_3CH_2CO_2$-$C_{3'}$), 59.04 ($OCH_3$), 50.17 ($NCH_2$), 30.77 ($NCH_2CH_2CH_2N$), 14.33 ($CH_3CH_2CO_2$-$C_{3'}$), 14.14 ($CH_3CH_2CO_2$-$C_5$). Anal. Calcd for $C_{27}H_{40}N_4O_{12}$: C, 52.94; H, 6.54; N, 9.15. Found: C, 53.17; H, 6.39; N, 8.90.

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