solvent density, and viscosity, can be obtained for particles with sizes even in the Ångstrom range.[31]

Received: March 9, 2001 [Z16745]

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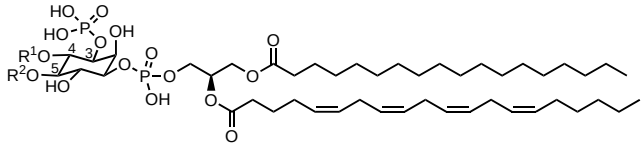
Membrane-Permeant 3-OH-Phosphorylated Phosphoinositide Derivatives**

Carlo Dinkel, Mark Moody, Alexis Traynor-Kaplan, and Carsten Schultz*

Phosphoinositides that are phosphorylated at the 3-hydroxy group (Scheme 1) are important signaling molecules in eukaryotic cells, particularly because of their participation in the receptor-mediated activation of protein kinases and in



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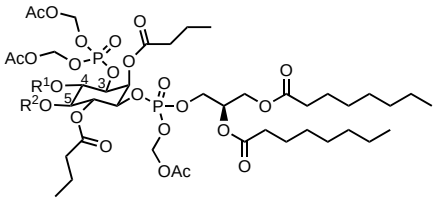

4–6

Scheme 1. Phosphoinositides PtdIns(3,5)P_2 (**1**): $R^1 = H$, $R^2 = P(O)(OH)_2$; PtdIns(3,4,5)P_3 (**2**): $R^1 = R^2 = P(O)(OH)_2$; PtdIns(3,4)P_2 (**3**): $R^1 = P(O)(OH)_2$, $R^2 = H$ and the corresponding membrane-permeant derivatives which can be bioactivated: di-C_8-Bt_3PtdIns(3,5)P_2/AM (**4**, $R^1 = Bt$, $R^2 = P(O)(OCH_2OAc)_2$), di-C_8-Bt_2PtdIns(3,4,5)P_3/AM (**5**, $R^1 = R^2 = P(O)(OCH_2OAc)_2$), and di-$C_8$-$Bt_3$PtdIns(3,4)$P_2$/AM (**6**, $R^1 = P(O)(O\text{-}CH_2OAc)_2$, $R^2 = Bt$). $Bt = COC_3H_7$.

the control of intracellular calcium levels. The key enzyme involved in the biosynthesis is the phosphoinositide 3-kinase (PI 3-kinase), which is activated by growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). The 3-hydroxy phosphorylated phosphoinositides formed by the enzyme have been associated with the mechanisms of oncogene transformation, cytoskeletal rearrangements, association of proteins to the plasma membrane, protein trafficking, endo- and exocytosis, the uptake of glucose by adipocytes, and the regulation of epithelial

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[**] This work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, as well as by the National Institutes of Health (SBIR NIH #R44 DK 52733-02 to A.T.-K.), and the Cystic Fibrosis Foundation, USA.

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chloride transport.[1, 2] The biosynthesis relies on various isoforms and splice variants of PI 3-kinase, and although usually more than one 3-OH-phosphorylated phosphoinositide is found in cells, most of them appear to originate from the same biosynthetic pathway.[1] PtdIns(3,5)P_2 seems to appear predominantly in yeast and to a lesser extent in mammalian cells. Because of its orphan status, PtdIns(3,5)P_2 is an interesting isomer for physiological tests.[3]

Which phosphoinositide connects the growth factor receptor signal to a physiological response, in our case to epithelial chloride secretion (Figure 1), the deficiency associated with

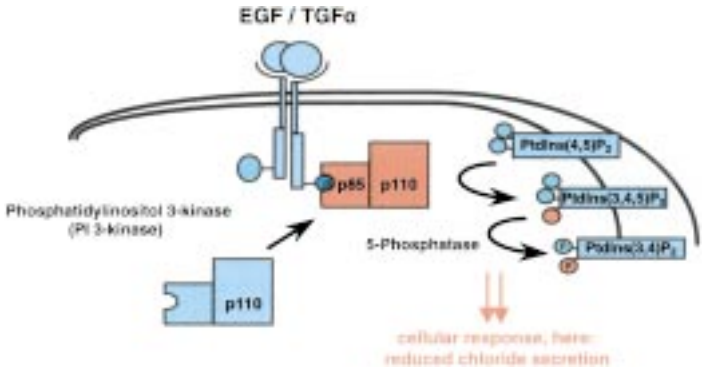
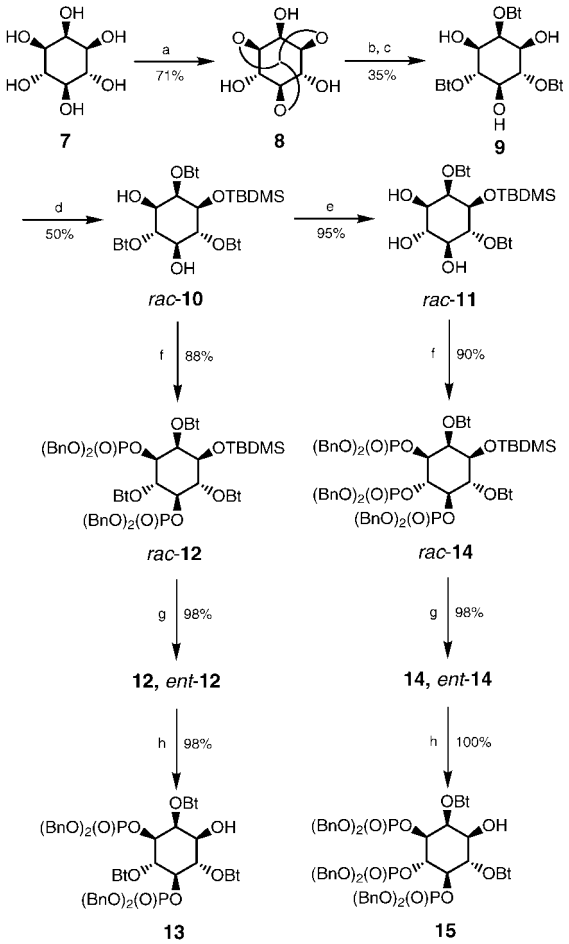


Figure 1. Partial growth factor signaling pathway: EGF or TGFα activate receptor tyrosine kinases, resulting in translocation of PI 3-kinase from the cytosol to the plasma membrane, where the heterodimeric enzyme complex p85/p110 phosphorylates phosphoinositides at the 3-OH group. Which of the resulting 3-OH-phosphorylated products mediates the signal towards the cellular response?

the deadly hereditary disease cystic fibrosis (CF)? To answer this question we synthesized membrane-permeant derivatives of three of the 3-OH-phosphorylated phosphoinositides, namely phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2, **1**), the trisphosphate PtdIns(3,4,5)P_3 **2**, and its metabolite PtdIns(3,4)P_2 **3** (Scheme 1). Membrane-permeant derivatives of inositol phosphates and phosphoinositides have previously been shown to be useful tools to mimic intracellular signals without disrupting the plasma membrane when administered.[2, 4, 5] The hydroxy groups of the previously used membrane-permeant PtdIns(3,4,5)P_3 derivatives were either not or only partially protected as butyrate esters.[2] However, the presumably slowly cleaved butyrate groups will prevent scrambling of phosphate groups by intramolecular migration[5] when the fast enzymatic hydrolysis of the acetoxymethyl ester groups takes place. This appears to be particularly important in the case of the closely related bisphosphate isomers **1** and **3**, which would otherwise be readily interconverted. Extracellular addition of the compounds should predominantly elevate the concentration of a single phosphoinositide when a short-term physiological response is observed. We therefore studied the ability of EGF and the three phosphoinositide derivatives to inhibit transepithelial chloride secretion of nasal epithelia from patients suffering from cystic fibrosis. It appears to be increasingly important to understand the growth factor mediated signaling in CF tissue, because there is increasing evidence that growth factor levels are greatly enhanced in lung tissue of CF patients.[6]

Similarly to the majority of syntheses of inositol phosphates, we started from *myo*-inositol (**7**). Tributyrate **9** was prepared in three steps via orthoester **8**.[7] Monosilylation of the more reactive 1-/3-hydroxy group led to *rac*-**10**, which served as a precursor in the synthesis of the PtdIns(3,4,5)P_3 and PtdIns(3,5)P_2 derivatives (Scheme 2). Phosphitylation of



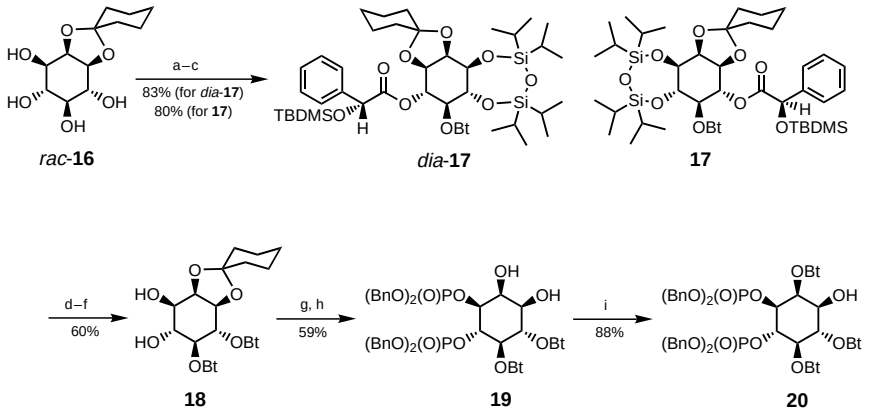
Scheme 2. a) HC(OMe)$_3$, PTSA, DMF, 120 °C, 18 h; b) Bt_2O, DMAP, pyridine, 25 °C, 18 h; c) TFA, H_2O, MeOH, 25 °C, 8 h; d) TBDMSCl, imidazole, DMF, 25 °C, 4 d; e) lipase from *Candida cylindracea*, 0.1 M phosphate buffer pH 8, MeOH, 25 °C, 5 d; f) $(BnO)_2PNiPr_2$, tetrazole, MeCN, 25 °C, 3 d, then $MeCO_3H$ (32 %), $-30 \rightarrow 25$ °C, 1 h; g) separation of enantiomers on Chiradex (Merck), eluent: 100 % MeCN; h) $Et_3N \cdot 3HF$, MeCN, 25 °C, 3 d. PTSA = *p*-toluenesulfonic acid, DMAP = 4-dimethylaminopyridine, TFA = trifluoroacetic acid, TBDMS = *tert*-butyldimethylsilyl, Bn = benzyl.

rac-**10** and subsequent oxidation of the phosphoric triester[8] resulted in the fully protected inositol 3,5-bisphosphate derivative *rac*-**12**. The enantiomers were separated by preparative chiral HPLC (50 × 250 mm, Chiradex, Merck)[9] and furnished the alcohols **13** and *ent*-**13** after cleavage of the silyl groups by treatment with triethylamine trihydrofluoride.

For the synthesis of the 3,4,5-phosphorylated precursor, we discovered that pig liver esterase and lipase from *Candida cylindracea* regioselectively cleaved the sterically less hin-

dered 4-O-butyrate group of *rac*-**10** in almost quantitative yield, but unfortunately not enantioselectively.[10] The resulting diester *rac*-**11** was phosphorylated and the enantiomers **14** and *ent*-**14** were separated subsequently by chiral HPLC. Deprotection gave the 1- and 3-hydroxy compounds **15** and *ent*-**15**, respectively, which are suitable for the lipid coupling.

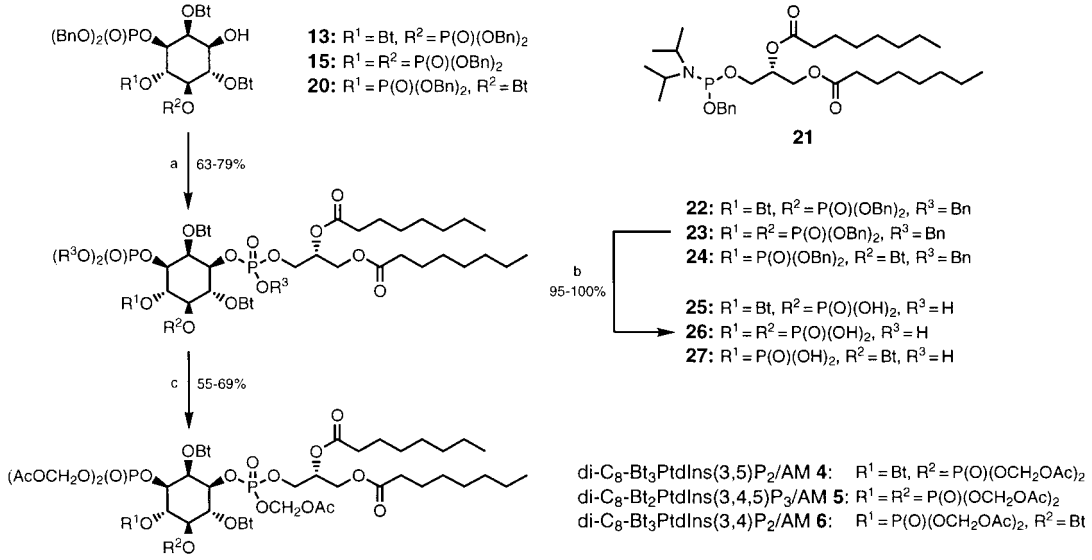
Early attempts to prepare enantiomerically pure precursors for the desired PtdIns(3,4)P_2 derivative **6** were set back by the discovery that a protected inositol bisphosphate similar to those described above did not separate on chiral HPLC. This hints towards the essential character of the 5-position in the molecular recognition by the chiral β-cyclodextrin resin. We therefore developed a facile three-step one-pot reaction to produce the diastereomeric mandeleic esters **17** and *dia*-**17** starting from monoketal *rac*-**16** (Scheme 3).[11] The diastereomers were separated on silica gel with a surprisingly high mass recovery (83 and 80%, respectively). Exchange of the chiral auxiliary for a butyryl group and subsequent desilylation gave dibutyrate **18**, which was subsequently phosphorylated. After removal of the cyclohexylidene ketal, the third butyryl group was introduced regioselectively at the axial 2-OH group of diol **19** via an intermediate orthobutyrate.[12] The spontaneous hydrolysis by stirring in water-containing methanol furnished the 1-hydroxy-inositol derivative **20** with high selectivity in 88% yield.



Scheme 3. a) TIPDSCl$_2$, pyridine, 25 °C, 3 d; b) (−)-2-*O*-TBDMS mandelic acid chloride, pyridine, −20 →25 °C, 1 d; c) Bt_2O, DMAP, pyridine, 80 °C, 5 d, separation of diastereomers on silica gel, petroleum ether (55–62 °C)/EtOAc 25:1, then 10:1; d) $MeNH_2$, EtOH, H_2O, 25 °C, 2 h; e) Bt_2O, DMAP, pyridine, 25 °C →60 °C; f) TBAF (5 equiv), TBME, 25 °C, 2 h; g) $(BnO)_2PNiPr_2$, tetrazole, MeCN, 25 °C, 3 d, then $MeCO_3H$ (32%), −30 →25 °C, 1 h; h) TFA, MeOH, 25 °C, 1 h; i) $MeCH_2CH_2C(OMe)_3$, PTSA, 25 °C, 2 h, then aq. MeOH (95%), 25 °C, 18 h. TIPDSCl$_2$ = 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, TBAF = tetrabutylammonium fluoride, TBME = *tert*-butyl methyl ether.

The final steps are shown in Scheme 4. Alcohols **13**, **15**, and **20** were coupled with the enantiomerically pure phosphoamidite **21**.[13] Subsequent oxidation with peracetic acid gave the fully protected phosphoinositide derivatives **22**–**24**, respectively. Removal of the benzyl groups by hydrogenolysis and alkylation of the free phosphate groups with acetoxymethyl bromide[14] led to the membrane-permeant phosphoinositide derivatives **4**–**6** as pairs of R_{P1}/S_{P1}-diastereomers in 5–15% overall yield. The absolute configurations were determined by the preparation of known reference *myo*-inositol derivatives from the enantiomerically pure precursors *ent*-**13**, **15**, and **17**, respectively (see Experimental Section for details).

For membrane-permeant derivatives of inositol phosphates and phosphoinositides it was previously shown that the bioactivated modifications of the hydroxy and phosphate groups allow diffusion over the plasma membrane of living cells, that the original highly charged compound is formed intracellularly, and that the biological activity of the messenger molecules can be monitored.[2, 4, 5] Phosphoinositides **4**–**6** provide us for the first time with the opportunity to distinguish whether the downstream signal transduction of growth factors



Scheme 4. a) **21**, tetrazole, CH_2Cl_2, 25 °C, 3 d, then $MeCO_3H$ (32%), −30 →25 °C, 1 h; b) H_2 (1 atm), Pd(C), $MeCO_2H$, 25 °C, 1 d; c) $AcOCH_2Br$, iPr_2NEt, MeCN, 25 °C, 3 d.

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(Figure 1) relies on PtdIns(3,4,5)P_3 or its metabolite PtdIns(3,4)P_2. This is of particular interest in cystic fibrosis, in which chloride secretion of epithelial cells is greatly diminished as a result of a point mutation in the cAMP-regulated apical chloride channel.[15] To overcome the resulting pathological conditions, the activation of alternative chloride channels, particularly those regulated by intracellular calcium, represents one of the possible therapeutic strategies.[16] However, these channels are under the negative control of several external signals including growth factors.[2, 17] Growth factors are abundant in the infected lung tissue of cystic fibrosis patients and are therefore suspected to hinder chloride secretion.[6]

Herein we show that EGF suppresses ATP-induced chloride secretion of nasal epithelial cells in CF patients. ATP is an agonist that stimulates calcium-mediated chloride secretion. When cells were preincubated with di-C_8-Bt_2PtdIns(3,4,5)P_3/AM (**5**; 200 μM) the negative effect of EGF on the chloride secretion was mimicked (Figure 2). In contrast, when cells

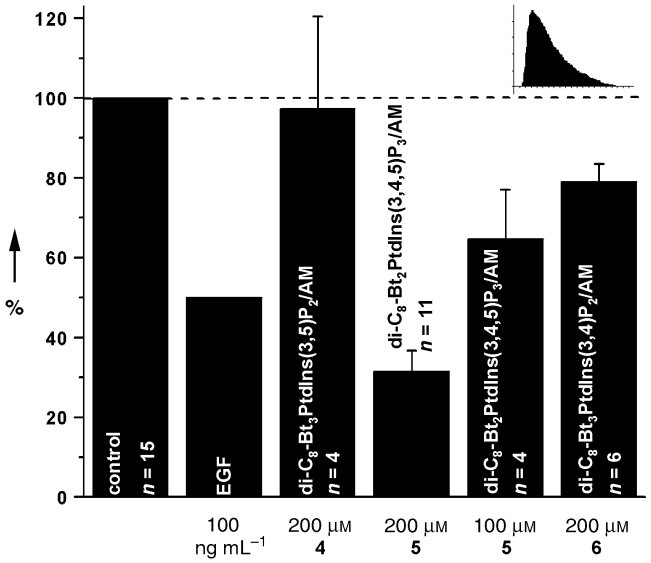

Figure 2. Effect of membrane-permeant phosphoinositide derivatives **4**–**6** on ATP-stimulated chloride secretion, measured as a short-circuit current (I_{SC}) of cultured human nasal epithelial cells from a cystic fibrosis patient: integral analysis (see insert) as percent of control.

were treated with the corresponding 3,5-bisphosphate **4** (200 μM), chloride secretion was in the range of the control experiments, whereas the 3,4-bisphosphate **6** showed only a marginally inhibitory effect. The results suggest that mainly PtdIns(3,4,5)P_3 (**2**) but not its metabolite **3** mediates the growth factor signal that limits calcium-mediated chloride secretion. These results make PtdIns(3,4,5)P_3-binding proteins prime targets for the development of PtdIns(3,4,5)P_3 antagonists that could potentially become a treatment against cystic fibrosis.

Experimental Section

Nasal epithelial tissue from CF patients was provided by the Children's Hospital in Seattle, USA. Cells were held in culture for up to four passages and were grown to confluency on permeable supports.

Ion-transport measurements: chloride secretion was monitored by changes in short-circuit current (I_{SC}) after cells were placed in Ussing chambers as described before.[18] Increases in chloride secretion were induced by adding 100 μM ATP to the apical side of the monolayer.

Delivery of phosphoinositides to the cytosol: cells were pre-incubated with the membrane-permeant phosphoinositides **4**–**6** for 45 min prior to mounting the cells. Butyryl groups and acetoxymethyl ester groups were previously shown to be readily removed when the compounds entered the cells by diffusion, but were moderately stable in the extracellular space.[4]

Selected physical data for **5** (R_{P1}/S_{P1}-diastereomers): ^{1}H NMR (600 MHz, [D$_8$]toluene, 20 °C): $\delta = 0.793$ (t, $J = 7.4$ Hz, 3H; CH_3), 0.804 (t, $J = 7.6$ Hz, 3H; CH_3), 0.817 (t, $J = 7.3$ Hz, 3H; CH_3), 0.820 (t, $J = 7.1$ Hz, 3H; CH_3), 0.822 (t, $J = 7.1$ Hz, 6H; $2 \times CH_3$), 1.050 (t, $J = 7.5$ Hz, 3H; CH_3), 1.097 (t, $J = 7.5$ Hz, 3H; CH_3), 1.087–1.232 (m, 32H; $16 \times CH_2$), 1.444–1.594 (m, 12H; $6 \times \beta$-CH_2), 1.732 (s, 3H; Ac), 1.741 (s, 3H; Ac), 1.767 (s, 3H; Ac), 1.768 (s, 3H; Ac), 1.779 (s, 3H; Ac), 1.780 (s, 3H; Ac), 1.782 (s, 3H; Ac), 1.784 (s, 3H; Ac), 1.800 (s, 3H; Ac), 1.875–2.009 (m, 4H; $2 \times \beta$-CH_2), 1.826 (s, 3H; Ac), 1.830 (s, 3H; Ac), 1.836 (s, 3H; Ac), 1.881 (s, 3H; Ac), 1.961 (s, 3H; Ac), 2.040–2.323 (m, 12H; $2 \times \alpha$-CH_2), 2.664 (ddd, $J = 17.5$, 8.1, 7.3 Hz, 1H; α-CH_2), 2.723 (ddd, $J = 17.6$, 7.8, 7.5 Hz, 1H; α-CH_2), 2.732 (ddd, $J = 17.5$, 8.2, 7.2 Hz, 1H; α-CH_2), 2.779 (ddd, $J = 17.6$, 8.3, 7.2 Hz, 1H; α-CH_2), 4.165 (dd, $J = 12.1$, 6.2 Hz, 1H, H_y-sn1), 4.178 (ddd, $J = 11.1$, 7.7, 5.8 Hz, 1H; H_y-sn3), 4.315 (ddd, $J = 11.3$, 7.1, 4.3 Hz, 1H; H_y-sn3), 4.333 (dd, $J = 12.2$, 6.4 Hz, 1H; H_x-sn1), 4.390 (dd, $J = 6.8$, 5.2 Hz, 2H; $2 \times H_x$-sn3), 4.407 (dd, $J = 12.3$, 3.8 Hz, 1H; H_y-sn1), 4.510 (dd, $J = 12.3$, 3.6 Hz, 1H; H_x-sn1), 4.853 (ddd, $J = 9.5$, 9.5, 4.7 Hz, 1H; H-5), 4.868 (ddd, $J = 9.6$, 9.6, 4.7 Hz, 1H; H-5), 4.911–5.013 (m, 4H; H_x-1, H_y-1, H_x-3, H_y-3,), 5.089 (ddd, $J = 9.7$, 9.7, 9.7 Hz, 1H; H-4), 5.105 (ddd, $J = 9.6$, 9.6, 9.6 Hz, 1H; H-4), 5.326 (dddd, $J = 5.7$, 5.7, 4.2, 4.2 Hz, 1H; H_y-sn2), 5.460 (dddd, $J = 6.2$, 5.2, 5.2, 3.8 Hz, 1H; H_x-sn2), 5.571 (dd, $J = 11.6$, 5.3 Hz, 1H, H-6), 5.593 (dd, $J = 11.3$, 5.4 Hz, 1H; H-6), 5.625 (dAB, $J = 5.5$ Hz, $\tilde{\nu}_A = 5.614$, $\tilde{\nu}_B = 5.636$, $J_{P,H} = 7.2$ Hz, 2H; OCH_2O (P-1)), 5.647 (dAB, $J = 5.5$ Hz, $\tilde{\nu}_A = 5.635$, $\tilde{\nu}_B = 5.659$, $J_{P,H} = 8.0$ Hz, 2H; OCH_2O (P-1)), 5.742–5.896 (m, 24H; $12 \times OCH_2O$), 6.207 (dd, $J = 2.9$, 2.9 Hz, 2H; H_x-2, H_y-2); ^{13}C NMR (50 MHz, [D$_8$]toluene, 20 °C): $\delta = 13.61$, 13.64, 13.94, 14.03, 14.38, 18.26, 18.34, 18.81, 20.22, 20.31, 23.15, 25.31, 29.54, 29.57, 32.22, 34.12, 34.18, 34.27, 34.36, 35.77, 35.81, 35.93, 36.07, 62.02, 62.29, 66.67, 69.58, 69.82, 70.03, 70.20, 73.72, 76.67, 76.98, 82.95, 83.06, 83.16, 83.47, 83.54, 168.85, 168.00, 169.17, 169.25, 169.32, 169.53, 172.14, 172.27, 172.57, 172.69, 172.90, 172.98, 173.08; ^{31}P NMR (81 MHz, [D$_8$]toluene, 20 °C): $\delta = -1.30$ (s, 1P), -2.10 (s, 1P), -2.84 (s, 1P), -2.86 (s, 1P), -3.13 (s, 2P), -3.77 (s, 2P); MS (DCI): m/z: 1397 $[M - CH_2OAc]^-$, 1143 $[M - C_{19}H_{35}O_4]^-$; HRMS (DCI, $R = 5000$) for $[M - CH_2OAc]^-$: calcd: 1397.37720, found: verified (±10 ppm).

Determination of absolute configurations: **4**: Compound *ent*-**13** was converted into *myo*-inositol 1,5-bisphosphate[19] by means of complete deprotection by hydrogenolysis and subsequent debutyrylation ($[\alpha]_D^{20} = +9.8°$, $c = 0.5$ in H_2O; lit.:[19] $[\alpha]_D = +6.0°$, $c = 0.5$ in H_2O). **5**: Compound **15** was converted into *myo*-inositol 1,3,4,5-tetrakisphosphate by phosphorylation of the 1-hydroxy group and complete deprotection ($[\alpha]_D^{20} = -3.2°$, $c = 1$ in H_2O; lit.:[20] $[\alpha]_D^{25} = -2.5°$, $c = 1$ in H_2O). **6**: Compound **17** was deacylated and desilylated to form 1,2-*O*-cyclohexylidene *myo*-inositol ($[\alpha]_D^{25} = -33.8°$, $c = 1$ in MeOH); lit.:[21] $[\alpha]_D^{22} = -36.0°$, $c = 1.05$ in MeOH, 96% *ee*).

Received: January 29, 2001
Revised: May 21, 2001 [Z16521]

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Synthesis and Spectral Characterization of a New Class of Heterocyclic Analogues of Crystal Violet Dyes**

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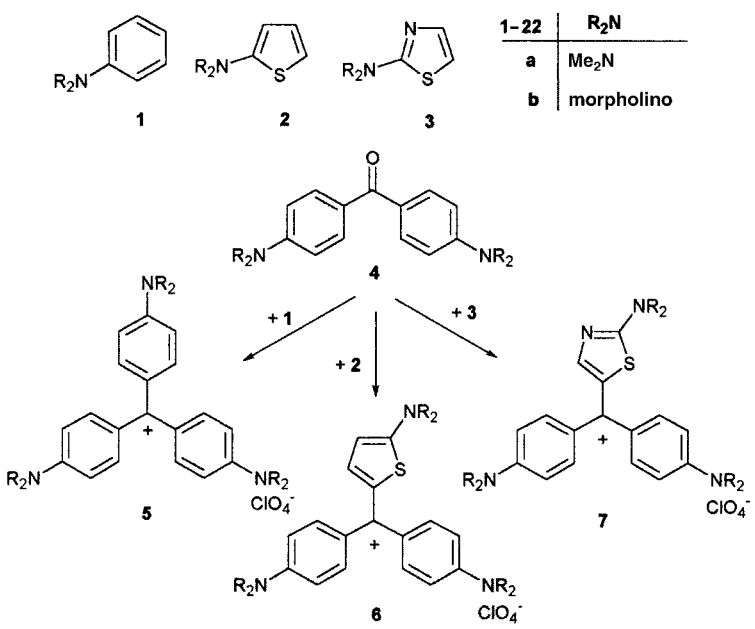
Dedicated to Professor Siegfried Hünig on the occasion of his 80th birthday

Crystal Violet **5a** is a classical representative of synthetic dyes that is to some extent still used today. It attracted considerable attention immediately after its discovery by von Hofmann in 1873, because such a beautiful and colorfast dye was unknown.[1] The dye can be synthesized by various methods usually starting from dimethylaniline (**1a**).[2] Some of these routes utilize Michler's ketone **4a** that is readily accessible from **1a** and phosgene (see Scheme 1).



Scheme 1. Synthesis of the Crystal Violet dyes **5**–**7**.

To improve the coloristic properties of Crystal Violet numerous experiments were performed to synthesize structurally modified derivatives. Among others, sulfonation leading to more water soluble, acidic Crystal Violet dyes, or the replacement of the benzenoidic rings by polycyclic or heterocyclic groups were tried succesfully. Further attempts to make the dyes more colorfast by substituting the N-alkyl groups by different types of aryl substituents were carried out.[3] With most of these dyes practical applications were rather limited, but from a theoretical point of view some of these compounds were of great importance.[4] A few years ago it was shown that Crystal Violet derivatives with an extended π system exhibit extraordinarily high quadrupol-induced nonlinear optical (NLO) coefficients through a multidirectional intramolecular charge transfer from the periphery to the center of the molecule that makes them promising candidates for the synthesis of materials with NLO properties.[5]

In recent years 2-dialkylaminothiophenes **2**[6] and 2-dialkylaminothiazoles **3**[7] have received special attention as heterocyclic analogues of the dialkylanilines **1**, since they are also able to form a large variety of different dyes. Thus, these heterocyclic amines can be used for, among other things, the preparation of diazo dyes,[8, 9] methine and azomethine dyes,[10, 11] as well as squaraine acid and croconine dyes.[12, 13] They can also be used for the preparation of a series of compounds that, because of their donor–acceptor character, possess an intense absorption in the visible spectral range and a high dipolarity. Thus, compounds of this type can be used as indicators for the determination of solvent polarities[14] or for the preparation of materials with NLO properties.[15] However, to our knowledge, compounds of type **2** and **3** have not

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[**] This work has been financially supported by the Deutsche Forschungsgemeinschaft.

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2001 1433-7851/01/4016-3008 $ 17.50+.50/0