4-(D-Glucosamino)-7-nitrobenzoxadiazole: synthesis, anomers, spectra, TLC behavior, and applications

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Abstract

A new synthesis of the known fluorescent compound **3** [4-(D-glucosamino)-7-nitro-2,1,3-benzoxadiazol-4-yl] is reported, starting from D-glucosamine and non-fluorescent 4-aryloxy-7-nitrobenzofurazans, **4a-e, 5**. The α and β anomers are easily interconverted but can be separated by TLC (R_f β > R_f α). The non-fluorescent new congener **8** {2-[N-(2',4',6'-trinitrophenyl)-amino]-2-deoxy-D-glucose} and the related known compound **9** {2-[N-(2',4'-dinitrophenyl)-amino]-2-deoxy-D-glucose} have anomers that may be seen in NMR spectra, but are too rapidly interconverted for TLC separation. The UV-Vis and fluorescence spectra of **3** depend markedly on solvent polarity. The TLC method allows the analytical determination of glucosamine from pharmaceutical preparations by conversion into **3** and detection by its fluorescence.

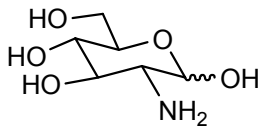
Keywords: D-Glucosamine-NBD, N-picryl-D-glucosamine, TLC behavior, fluorescence, conformational studies, analytical determination of glucosamine

Introduction

The strong fluorescence of 4-nitrobenzofurazans (NBD) having an aminic nitrogen atom in the 7-position has contributed to the wide use of such compounds as biological test reagents.[1-8] Moreover, some of these derivatives have antileukemic or immunosuppressive properties.[1,2,9]

Glucosamine (2-amino-2-deoxy-D-glucose) (**1**) is one of the constituents of chitin, chitosan, and mucopolysaccharides and has important biomedical applications, including pharmaceutical preparations for treating cartilage diseases of joints.[10-14] Such preparations include often glucosamine hydrochloride or sulfate and chondroitin sulfate.

The fluorescent reaction product between 7-chloro-4-nitrobenzofurazan, (**2**, NBD-Cl) and glucosamine was described: 2-(N-(7'-nitrobenz-2'-oxa-1',3'-diazol-4'-yl)-amino)-2-deoxy-D-glucose[15,16] (NBD-NH-Glc, **3**). Initially, although ^{1}H-NMR data showed the presence of two distinct α and β anomers, only one TLC spot was detected.[15] More recently, it was possible to provide evidence for the separated anomers by HPLC.[16] The mixture of anomers **3** is commercially available for visualizing the transport and cellular metabolism of glucose and its analogs,[8,15-33] and for the electrophoretic determination of glucosamine.[34]



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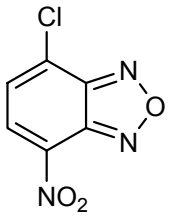

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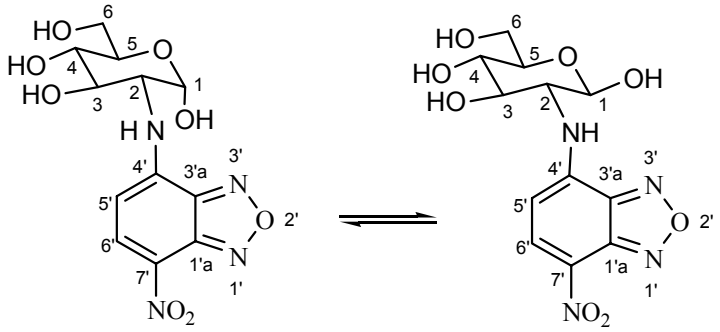
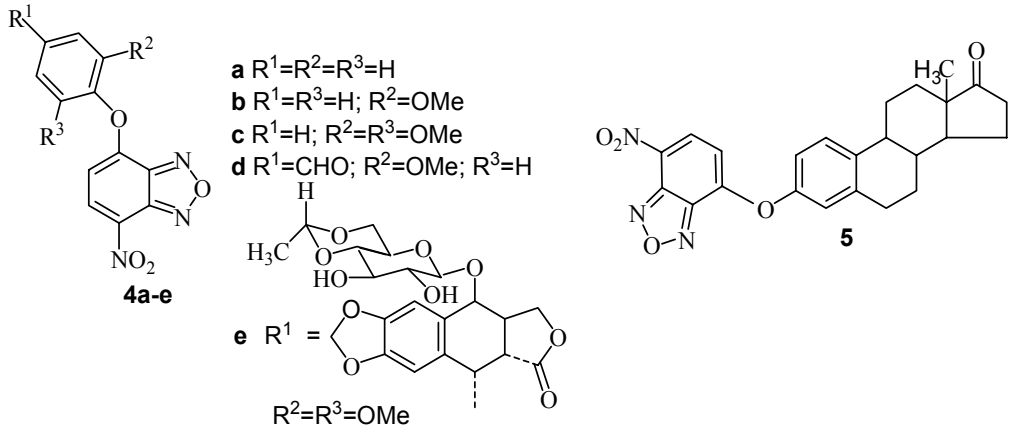
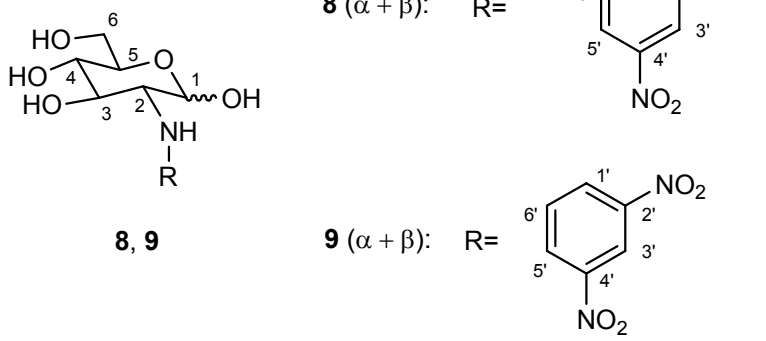
In the present communication we describe: (i) the TLC separation of the two anomers of **3** synthesized from **1** and **2**; (ii) another synthesis of **3** starting from **1** and various non-fluorescent 4-aryloxy-7-nitrobenzofurazans **4a-e, 5** [35-37] (ArO-NBD); (iii) the synthesis and characterization of 2-[N-(2',4',6'-trinitrophenyl)amino]-2-deoxy-D-glucose **8**, a new analog of the known 2-[N-(2',4'-dinitrophenyl)amino]-2-deoxy-D-glucose **9**,[38-43] which is a bioanalytical reagent for the spectrophotometric investigation of glucosamine transport and metabolism. The latter compounds **8** and **9** are non-separable mixtures of α and β anomers, and the synthesis was based on glucosamine and 2,4,6-trinitrochlorobenzene **6**, or 2,4-dinitrofluorobenzene, **7**. All compounds have been characterized by elemental analysis, TLC data, RP-TLC (for hydrophobic/hydrophilic balance), NMR, UV-Vis and fluorescence spectra and the solvent effects on all these data.



3



4a-e **5**



8, **9**

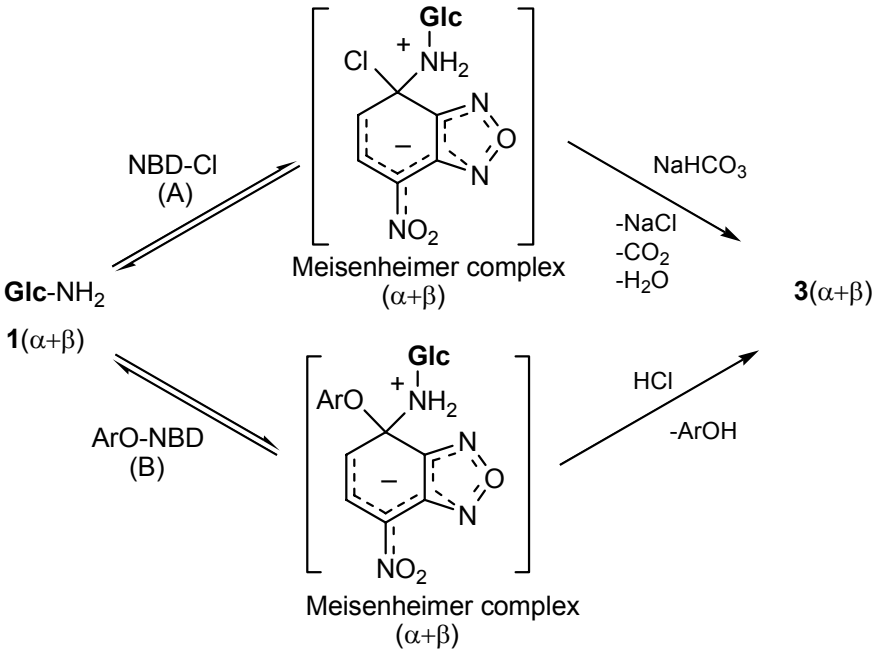
Results and Discussion

Synthesis of compounds 3, 8, and 9

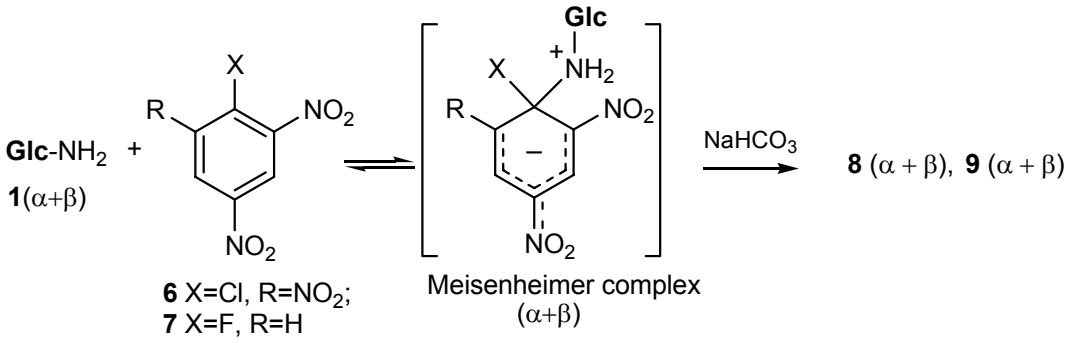
Two synthetic variants, A and B, were adopted for the formation of **3** (Scheme 1). In variant A, the reaction of **1** (as hydrochloride) and **2** takes place in the presence of sodium hydrogen carbonate in methanol at 50ºC, but the difference from the literature[15,16] occurs in the isolation and purification via preparative TLC (yield 40%). In variant B (yield 60%), based upon the

previous experience with amino acids,[37] the starting materials were **1** (as hydrochloride) and 4-aryloxy-7-nitrobenzofurazans **4a-e** or **5**,[35-37] at 30ºC, also in the presence of $NaHCO_3$. In both cases the reactions take place in an S_NAr process (Scheme 1) via a deeper-colored Meisenheimer complex.

Like the NBD moiety, polynitrophenyl groups are strongly electron-attracting and they were the prototype for the formation of Meisenheimer complexes.[35-37,44] By reacting the nucleophile **1** (as hydrochloride) with picryl chloride **6** in the presence of $NaHCO_3$, the new compound **8** was formed (Scheme 2). In order to obtain in an analogous way the known compound **9**,[38-43] the electrophile was 2,4-dinitrofluorobenzene, **7**. Although both these compounds give rise to anomers as attested by NMR evidence, TLC does not allow their separation.



Scheme 1. Synthesis of compound **3** by variants A and B.



Scheme 2. Synthesis of compounds **8** and **9.**

NMR Spectra

The ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 with trifluoroacetic acid (the latter allows a better resolution of hydroxyl protons). In the ^{1}H-NMR spectra of **3**, characteristic peaks for the NBD moiety correspond to aromatic protons H-6' and H-5', and for the glucosamine moiety to protons bonded to carbons 2, 3, 4, 5, and 6. The *α* anomer is characterized by the H-1 doublet at 5.23 ppm and the *β* anomer by the H-1 doublet at 4.78 ppm, similarly to the reported values in deuterium oxide (for *α,* δ = 5.27 ppm, and for *β,* δ = 4.78 ppm).[15,16] The relative amounts at equilibrium are *α* / *β* ≈ 1/1, similarly to literature data[15] (per cent ratio *α* / *β* = 42/58). In the ^{13}C-NMR spectra, characteristic peaks for the NBD moiety correspond to aromatic carbons (C-1'a, C-3'a, C-4',5',6',7') and for the glucosamine moiety to all six sp^3-hybridized carbon atoms.[45]

In the ^{1}H-NMR spectra of **8**, characteristic peaks for the NBD moiety correspond to aromatic protons H-3' and H-5', and for compound **9** to aromatic protons H-3',5', 6'. In both cases, the H-2,3,4,5,6 peaks are practically identical. For the *α* anomer of **8**, the H-1 doublet appears at 5.13 ppm, and for the *β* anomer at 4.79 ppm. For the *α* anomer of **9**, the H-1 doublet appears at 5.23 ppm, and for the *β* anomer at 4.63 ppm. Interestingly, the percent ratio for the two anomers differs markedly: *α*/*β* ≈ 95/5 for **8** and *α*/*β* ≈ 85/15 for **9**. The decreasing *α*/*β* anomer ratio **8** > **9** > **3** can be explained by the global electronegativity of the aromatic group which decreases in the same order, and also by the decreasing possibility for an intramolecular hydrogen bond between the axial *α*-hydroxy group and the nitro-O atom or the heterocyclic N-atom of the aromatic moiety.

Conformational studies for anomers 3α, 3β, 8α, 8β, 9α and 9β

It is well known that the interconversion of glucose anomers (mutarotation) is a general acid-base-catalyzed reaction which requires the simultaneous presence of a base and an acid.[46,47] With mineral acids or bases, the protic solvent provides the missing third partner. However, with phenols as acids and pyridines as bases, which do not undergo neutralization, it was proven that when both are present a marked acceleration occurs. An even higher acceleration occurs with 2-pyridone derivatives because a bimolecular process replaces the termolecular encounter.

In our case the equilibration of the two anomers is facilitated by the presence of the relatively acidic NH group so that one has to assume that the *α*/*β* anomer ratio always corresponds to the equilibrium ratio.

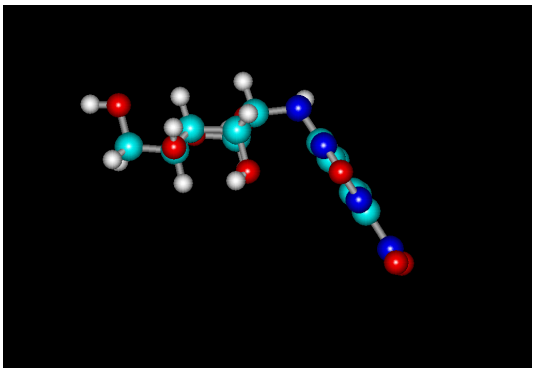
In order to better understand these anomer equilibria, theoretical conformational calculations were undertaken. The molecular geometry was determined by using the Hyperchem program,[48] and the results of the optimization are presented in Fig. 1. The energies computed with programs WinMOPAC 7.21 [49] and CODESSA [50] are displayed in Table 1.

Table 1. Energies of α and β anomers of compounds **3, 8** and **9**

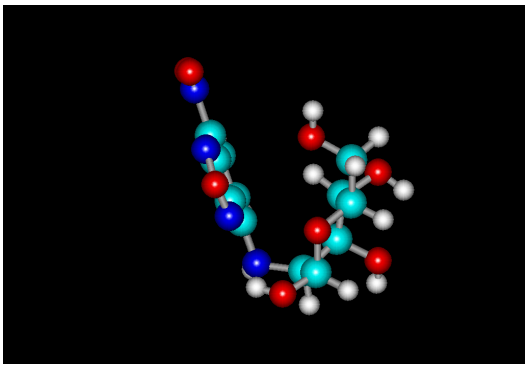
Energy (kcal/mol)	Compounds					
	3α	**3β**	**8α**	**8β**	**9α**	**9β**
Electronic energy[a]	-811514.79	-806423.31	-1042354.43	-1042549.79	-875570.54	-876014.66
Zero point vibrational energy[b]	4289.14	4288.90	4479.39	4836.15	4421.05	4763.96
HOMO-Energy[b]	-231.19	-230.49	-237.06	-226.82	-224.70	-220.74
LUMO-Energy[b]	-52.33	-52.37	-46.49	-42.18	-33.60	-35.21
Energy[a]	-118349.59	-118350.57	-141142.78	-141142.95	-121947.24	-121947.47

[a] WinMOPAC 7.21.[49] [b] CODESSA.[50]

It can be seen that the two anomers of **3** have higher energy differences ($\Delta E_T = E_T\beta - E_T\alpha$) than the other compounds: $\Delta E_{T(3)} = -0.98$ kcal/mol, $\Delta E_{T(8)} = -0.17$ kcal/mol, $\Delta E_{T(9)} = -0.23$ kcal/mol. This fact may provide an explanation for the fact that TLC is able to separate only the anomers of **3**.



3α



3β

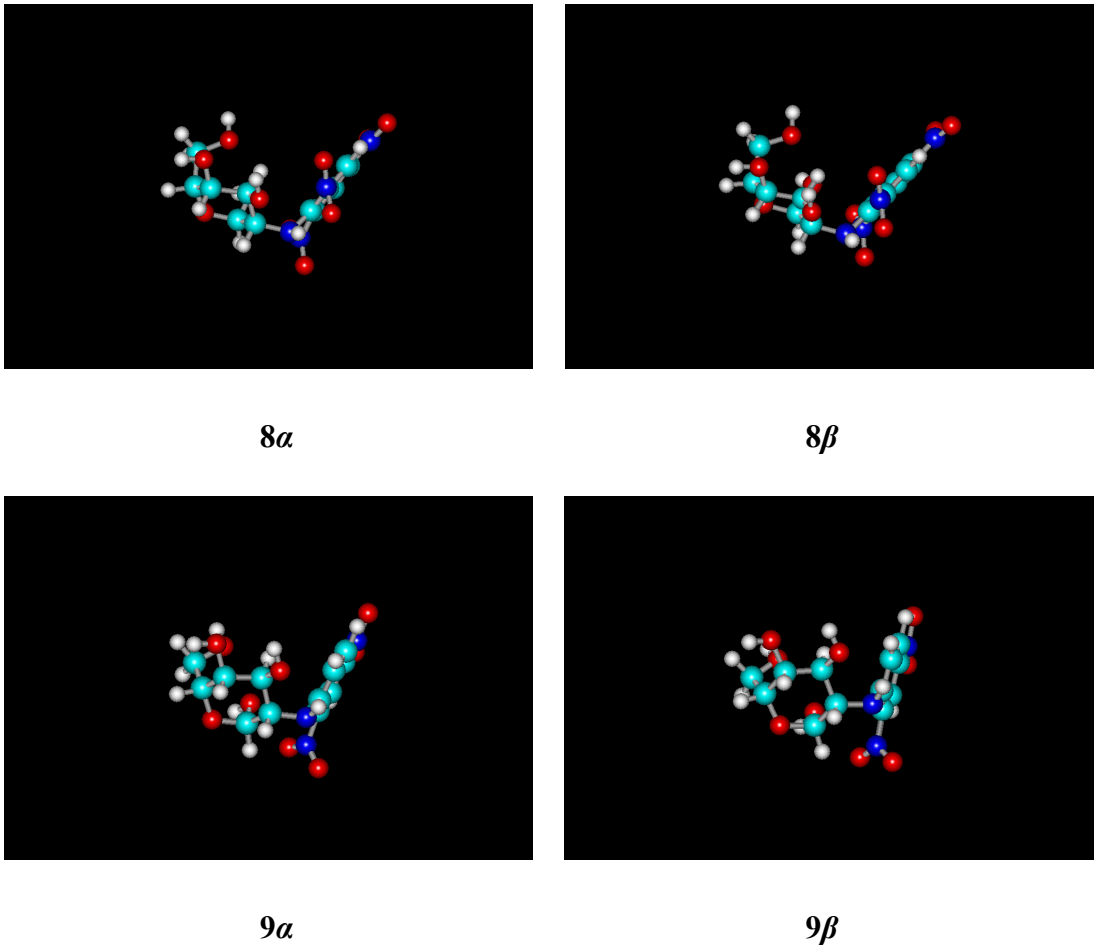
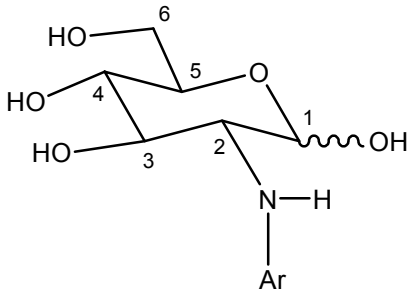


Figure 1. Optimized structures (with the MM+ force field from the Hyperchem[48] program) for *α* and *β* anomers corresponding to compounds **3**, **8** and **9**.

According to the optimized geometry from Figure 1, the *α* anomer of **3** has an "open" structure favoring stronger interactions with a flat surface, whereas the *β* anomer has a "closed" structure resembling a sandwich with nearly parallel rings. On considering the possibility of an intramolecular hydrogen bond between the NH group and the glycosidic oxygen for compounds **3**, **8**, and **9** by means of programs Hyperchem [48] and ArgusLab,[51,52] it was found (Table 2) that the O•••N distance in the **3***β* anomer is the lowest (closest to 3 Å);[53] *α* anomers have larger distances. These results may throw light on the differences in retention times due to interactions with Si–OH groups of the silica gel stationary phase.

Table 2. Distance between the nitrogen atom and the glycosidic oxygen computed by means of the Hyperchem program



3(α,β) Ar=NBD
8(α,β) Ar=Picryl
9(α,β) Ar=2,4-Dinitrophenyl

Compound	Distance (Å)
3α	3.617
3β	2.954
8α	4.463
8β	3.098
9α	4.424
9β	3.088

Thin-layer chromatographic data for anomers of 3

Room-temperature separations by TLC involving silica gel and a mixture of dichloromethane and methanol in three different ratios, presented as (a) – (c) in Table 3, indicated that compound **3** presented two spots (X and Y) irrespective of the synthesis method (A or B), whereas compounds **8** and **9** always gave rise to only one spot, with retention times R_f **8** ($\alpha + \beta$) > R_f **9** ($\alpha + \beta$) > R_f **3** X > R_f **3** Y.

Table 3. TLC behavior (R_f)[a,b] of compounds **3**,[c] **8**, and **9**

Compound	R_f(a)	R_f (b)	R_f(c)
3 X[d]	0.22	0.41	0.65
3 Y[d]	0.11	0.30	0.51
8 ($\alpha + \beta$)[e]	0.42	0.63	0.88
9 ($\alpha + \beta$)[e]	0.31	0.52	0.77

[a]Silica gel GF_{254} (Merck), with (a): methylene chloride: methanol (9:1, v/v); (b): methylene chloride: methanol (8.5:1.5, v/v); (c): methylene chloride: methanol (8:2, v/v). [b]At room temperature. [c]Obtained by **A** or **B** procedure (Scheme 1). [d]Detection by fluorescence (360 nm). [e]Detection at 254 nm.

By using a liquid–liquid partition (silica gel having a covalently-bonded hydrocarbon C_{18} chain and aqueous ethanol at three concentrations) it was possible to determine both the TLC

behavior and the hydrophobicity parameter R_{M0}.[54-57] The results are presented in Table 4. The statistical parameters *R*, *F*, and the standard deviation (*SD*) for R_{M0} are good to excellent.

Table 4. Experimental hydrophobicity (R_{M0} [a,b]) and calculated (log P)[58] for compounds **3, 8** and **9**

Comp.	Experimental data								Calcd. log P
	R_M in aqueous ethanol, conc.(v/v)			R_{M0}	*b*	Statistical parameters			
	70%	60%	50%			*R*	*F*	*SD*	
3	–0.5006	–0.6434	–1.0787	–2.475	0.029	0.959	11.72	0.119	–0.540
8	0.3273	–0.0321	–03521	–2.057	0.034	0.999	892.1	0.016	–0.280
9	0.1047	–02303	–0.5228	–2.098	0.031	0.999	653.9	0.017	–0.410

[a]Average for five determination on silica gel RP-18 F_{254} (Merck) with mixtures of ethanol:water; R_{M0} = molecular hydrophobicity, where R_{M0} is the R_M value of the organic component extrapolated to zero concentration in the organic:water mixture; *b* = the change in the R_M value caused by increasing the concentration (*K*) of the organic component in the mobile phase; *R* = the correlation coefficient for parameters R_{M0} and *b* described by ecuation $R_M = R_{M0} + bK$ [where $R_M = \log(1/(R_f\text{-}1))$].[54-57]

The results show that under these conditions, all three compounds migrate as an inseparable mixture of the two anomers; the hydrophobicity decreases in the order R_{M0} **8** > R_{M0} **9** > R_{M0} **3**, in agreement with the order for the α/β anomer ratio and with the known hydrophobicity of nitro groups (**trinitro**phenyl-aminodeoxyglucose, **8** > **dinitro**phenyl- aminodeoxyglucose, **9** > **nitro**benzodioxazolyl-aminodeoxyglucose, **3**).

On studying the partition in a solid–liquid system when the solvent is aqueous acetonitrile (17:3 v/v) as mentioned in the literature for **3**,[15,16] a single spot was observed. When there is no water in the mobile phase for stationary phase I, and water for stationary phase II, as in Table 5, two spots can be observed. This is in agreement with the idea that the equilibration **3**$\alpha \rightleftharpoons$ **3**β occurs rapidly in the aqueous mobile phase due to the general acid-base catalysis.

Since reaction rates are also strongly influenced by the temperature, TLC studies in ethyl acetate at various temperatures with two types of stationary phases (I and II) were carried out and the results are displayed in Table 5. The stationary phase I is the "classical" silica gel, whereas the stationary phase II is silica gel-C_{18} associated with a copper salt and a chiral derivative for use in separating enantiomers of amino acids.[59-61]

Table 5. TLC data of the two spots X and Y (*β* and *α*) for **3** synthesized by variant A

	Temperature (ºC)		R_f			% [e]		
TLC [a]	For **3** in ethyl acetate [b]	For mobile phase [c]	X(*β*)	Y(*α*)	ΔR_f [d]	X(*β*)	Y(*α*)	X(*β*)/Y(*α*)
I	24.2	20.7	0.21	0.13	0.08	65.05	34.87	1.86
	5.4	20.7	0.28	0.20	0.08	66.08	33.85	1.95
	5.5	4.75	0.46	0.34	0.12	65.81	34.08	1.93
	5.4	0.15	0.52	0.38	0.14	66.07	33.73	1.95
	5.7	–6.7	0.93	0.69	0.24	60.08	39.87	1.50
II	23.8	22.9	0.47	0.36	0.11	40.87	59.08	0.69
	5.6	14.9	0.50	0.35	0.15	38.82	61.10	0.63

[a]**I**: TLC analytical plates silica gel GF_{254} (Merck), CH_2Cl_2:MeOH 8.5:1.5 v/v, front mobile phase = 5cm; **II**: HPTLC analytical plates CHIR (Merck), H_2O:EtOH 9:1, front mobile phase = 5cm. [b]15 minutes at the indicated temperature. [c]Average value (between start and end of the development of the chromatographic determination). [b,c]Measured with digital termometer Diplex (–40ºC to 200ºC). [d]$\Delta R_f = R_f X - R_f Y$. [e]Quantitative (densitometric) TLC analysis (λ_{max}=341nm).

Densitometric TLC determinations show that on summing the chromatographic peak areas, the purity (> 99%) is confirmed. The data presented in Table 5 indicate that for the stationary phase I the spot with higher R_f (X) corresponds to the β anomer, which prevails in the equilibrium mixture due to its lower steric hindrance and energy. Recently, literature HPLC data revealed a similar elution time order: t_R β > t_R α.[16] Our data presented in Table 5 show the influence of lower temperatures regarding the chromatographic resolution for the anomers α and β (the ΔR_f value).

For the stationary phase II, due to interactions with the copper complex (known in the case of glucose),[62-64] the order of migration of the two anomers is not so clear; we assume that the order in which the two anomers migrate becomes reversed, and that the *β*/*α* ratio does not conserve the prevalence of the *β* anomer in the equilibrium mixture.

The behavior of **3** obtained by synthetic variant B is similar to that via variant A: the purity is again > 99% by densitometric analysis on summing the areas of the two peaks.

UV-Vis and fluorescence spectra of compound 3 (α + β)

Compound **3** in crystalline state and in solution is yellow-orange and strongly fluorescent. The longest-wavelength absorption band presents in various solvents a positive solvatochromy: λ_{max} increases with increasing values of Reichardt's empirical solvent polariy parameter,[65] as one can observe from Table 6.

The fluorescence characteristics, namely λ_{ex}, λ_{em}, the quantum yield Φ, natural lifetime τ_0 and calculated lifetime τ, also depend on the solvent polarity: with increasing $E_T(30)$, Φ and *τ*

(which includes parameter Φ) decrease. From Figure 2, one can see that in aqueous ethanol the fluorescence intensity decreases with decreasing ethanol concentration. Also, with decreasing ethanol concentration, one observes a decrease of the fluorescence parameters Φ, τ_0 and τ, in agreement with qualitative literature data about the fluorescence of **3** *(α + β)* in water.[16]

These findings are useful for applications of **3** as a fluorescent probe for assaying glucosamine, because the adequate choice of solvent is critical for obtaining a satisfactory intensity of fluorescence.

Table 6. UV-Vis spectral data and the fluorescence characteristics: λ_{ex}, λ_{em}, quantum yield (Φ), natural lifetime (τ_0) and calculated lifetime (τ) in various solvents for compound **3** ($\alpha + \beta$)[a,b]

Solvent ($E_T(30)$)[65]	λ_{max} (nm)	$\varepsilon \times 10^3$ (L×mole^{-1} × cm^{-1})	λ_{ex} (nm)	λ_{em} (nm)	Φ[e]	τ_0 (ns)[f]	τ (ns)[g]
Ethyl acetate (38.1)	274.2 326.0 sh[d] 456.4	22.74 10.75 22.98		520.3	0.105	8.00	0.84
Ethanol[c] (51.9)	267.3 330.7 466.3	3.17 10.02 24.69	450	532.4	0.0522	7.98	0.41
Ethanol[c] :water 8:2 v/v (53.7)	269.6 334.2 469.2	3.42 11.73 31.78		535.1	0.0265	6.46	0.17

[a]Obtained by A or B procedure (Scheme 1); [b]concentration of **3** = 4.09×10^{-5}M. [c]Absolute ethanol; [d] sh=shoulder. [e]Compared to quinine bisulfate (in 0.1N H_2SO_4, Φ=0.55). [f]Estimated using the Strickler-Berg formula[66]: $\frac{1}{\tau_0} = 2.88\times10^{-9} n^2 \frac{\int I_F(\nu_F)d\nu_F}{\int I_F(\nu_F)\nu_F^{-3}d\nu_F} \times \int \frac{\varepsilon(\nu_A)}{\nu_A} d\nu_A$

where: τ_0 is the lifetime, ν is the wavenumber of the maximum absorption band, n is the refractive index of the solvent (1.3595 for ethanol), I_F is the fluorescence intensity and ε, the molar absorption coefficient. [g]$\tau = \tau_0 \times \Phi$.

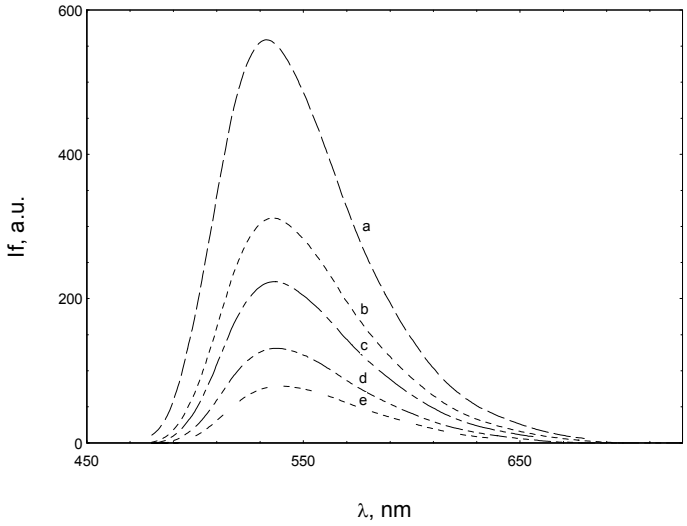


Figure 2. Change of the fluorescence intensity (λ_{ex}=450 nm) for compound **3** (*α* + *β)*: (concentration = 5.84×10^{-5}M) in: a = ethanol; b = 80% ethanol-water; c = 60% ethanol-water; d = 40%ethanol-water; e =2 0%ethanol-water.

Analysis of glucosamine in pharmaceutical preparations

As mentioned earlier, salts of D-glucosamine with hydrochloric or sulfuric acid are used therapeutically for treatment and functional maintenance of cartilage in joints, and under the form of nutritional supplements.[10,67] Often they are associated with chondroitin sulfate.[67,68]

For analytical and bioanalytical qualitative or quantitative determinations of **3** (*α* + *β)* [8,15-33] one may use TLC as a simpler alternative to electrophoresis [34] or HPLC.[16] The procedure involves reacting 5 to 10 mg of powdered pharmaceutical preparation either with **1** or with **4a**, in conditions described in the Experimental Part, followed by TLC analysis for qualitative analysis (one or two spots depending on the adsorbant and solvent) or by quantitative densitometry when the detection involves UV-Vis or fluorescence spectroscopy.

Conclusions

NMR spectra of the fluorescent compound **3** (synthesized by a novel method using glucosamine **1** and 7-aryloxy-4-nitrobenzofurazans **4** or **5**) provided evidence for the two *α* and *β* anomers. They could be visualized by TLC under special conditions of solid phase and solvent mixture. The less sterically hindered *β* anomer is present in slighly higher concentration in the equilibrium mixture. By contrast, the *α* and *β* anomers of analogous polynitrophenyl-substituted compounds **8** and **9** cannot be separated by TLC although their presence is proved by NMR spectra; in this case, probably owing to the higher electronegativity and better hydrogen-bond acceptor properties of *ortho*-situated nitro groups, the *α* anomer appears to predominate in the equilibrium

mixture. The solvent polarity influences markedly the UV-Vis and fluorescence spectra of **3** (α + β). The TLC method may be applied for determining the presence of glucosamine in pharmaceutical preparations.

Experimental Section

General Procedures. Commercial products were employed: D-glucosamine hydrochloride **1** (Acros Organics), **2** (Aldrich), **4a-e** and **5**,[35-37] **6** (Merck), **7**,[69] TLC analytical silica gel plates GF_{254}, TLC preparative silica gel plates PLC- F_{254}, TLC analytical silica gel plates RP-18F_{254} and HPTLC analytical plates CHIR (Merck).
^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Varian Gemini 300BB spectrometer (300MHz for ^{1}H and 75MHz for ^{13}C). We used Camag Software 1992 scanner II – Switzerland for densitometric TLC analysis. Temperatures were recorded with a digital termometer Diplex (-40ºC to 200ºC). The absorption spectra were recorded with Perkin Elmer Lambda 35 UV-vis spectrometer; conditions are specified in Table 6. For fluorescence spectra, a Perkin-Elmer 204 spectrofluorimeter was used; conditions are specified in Table 6. For fluorescence, an excitation lamp (Xe, 150 W) interfaced with the computer was used, allowing a pre-established data reading time of 0.5 s; IR spectra were recorded with FTIR spectrophotometer Bruker-Model Vertex 70, using ATR techniques. Melting points have been recorded in open capillary with Electrothermal IA 9000 Series of digital melting point instruments.

Synthesis of compounds 3 (α + β), 8(α + β) and 9(α + β). General procedure
Variant A was applied for obtaining compounds **3, 8**, and **9**. Starting from D-gluocosamine **1** (as hydrochloride) and halogen derivatives **2**, **6**, **7** (molar ratio 1:1) in methanol (5 mL for one gram of reactant mixture) and sodium hydrogen carbonate (2.5 moles for each mole of reactant **1**) the reaction mixture was stirred at 50ºC (for **3** during 1 h, for **8** during 24 h, and for **9** during 7 days). The reaction mixture was filtered and the filtrate was evaporated under vacuum. The residue was purified by preparative TLC using preparative silica gel TLC plates PLC- F_{254} (Merck): for **3**, the mobile phase was dichloromethane: methanol 8.5:1.5, v/v; for **8** and **9**, dichloromethane: methanol 9:1, v/v. Fluorescence detection was used at 360 nm for **3**, and at 254 nm for **8** and **9**. The area of maximum concentration was retained and extracted in a Soxhlet with dichloromethane:methanol (9:1, v/v), followed by evaporation under vacuum. With TLC using silica gel GF_{254} (Merck) analytical plates, methylene chloride:methanol 8.5:1.5 v/v, two spots were detected by densitometric analysis.
Variant B was used only for **3** (α + β) starting from D-glucosamine **1** (as hydrochloride) and **4a** (molar ratio 1:1.2) in the presence of $NaHCO_3$ in methanol (5 g for one gram of reactant mixture) at 30˚C for 24 h. The reaction mixture was then worked up as indicated above, and the product **3** was indistinguishable from that obtained by variant A. With compounds **4b** – **4e** or **5**, similar results were obtained.

2-[*N*-(7'-Nitrobenz-2'-oxa-1',3'-diazol-4'-yl)amino]-2-deoxy-D-glucose (3) (α + β). Orange-colored, 40% yield (variant A), or 60% (variant B), m.p. 159-160ºC; Anal.: Calcd. for $C_{12}H_{14}N_4O_8$: C 42.11; H 4.12; N 16.36; found C 41.9; H 4.8; N 16.30%. ^{1}H-NMR (in dmso-d_6+TFA, δ ppm, *J* Hz): 9.42(bs, 1H, NH); 8.50(d, 1H, 9.1, H-6'); 6.90(d, 1H, 9.1, H-5'); 5.23(d, 0.5H, H-1α, 3.2); 4.78(d, 0.5H, H-1β, 8.6); 4.07-3.48(m, 4H, H-2-3-4-5); 3.36-3.14(m, AB system, 2H, H-6A and H-6B). ^{13}C-NMR (dmso-d_6+TFA, δ ppm): 147.05(C-7'); 145.14(C-4'); 144.81(C-1'a); 138.42(C-6'); 120.80(C-3'a); 100.88(C-5'); 77.53(CH-1); 75.14(CH-3); 72.92(CH-4); 71.93(CH-5); 71.14(CH-2); 61.58(C-6).

2-[*N*-(2',4',6'-Trinitrophenyl)amino]-2-deoxy-D-glucose 8 (α + β). Yellow, 50% yield, m.p. 103-104ºC; Anal.: Calcd. for $C_{12}H_{14}N_4O_{11}$: C 36,93; H 3.61; N 14.35; found C 36.90; H 3.58; N 14.31%; IR (solid ATR), cm^{-1}: 1590, 1618 (NO_2), 2852, 2922 (CH, CH_2), 3295 (OH); ^{1}H-NMR (dmso-d6+TFA, δ ppm, *J* Hz): 8.92(bs, 2H, H-3'5'); 5.23(d, H-1α, 3.4); 4.63(d, H-1β, 7.7); 3.64-3.42(m, 3H, H-3-4-6A); 3.10-3.03(m, 2H, H-2-5); 3.00(dd, AB system, H-6B, 3.5, 9.7). ^{13}C-NMR (dmso-d_6, δ ppm): 143.01(C-1'); 138.87(C-2'-6'); 133.50(C-4'); 127.09(C-3'-5'); 89.29(CH-1); 73.71(CH-3); 72.56(CH-4); 69.56(CH-5); 61.30(CH-2); 60.58(CH_2-6). ^{13}C-NMR (dmso-d_6+TFA, δ ppm): 143.29(C-1'); 137.69(C-4'); 133.73(C-2'-6'); 127.23(C-3'-5'); 89.56(CH-1); 73.96(CH-3); 72.78(CH-4); 69.81(CH-5); 61.54(CH-2); 60.81(CH_2-6).

2-[N-(2',4'-Dinitrophenyl)amino]-2-deoxy-D-glucose 9 (α + β). Yellow, 46% yield, m.p. 192-194ºC (lit. 194-196ºC [38,39] and 201-203ºC [38,39]); Anal.: Calcd. for $C_{12}H_{15}N_3O_9$: C 41,74; H 4.37; N 12.17; found C 41.71; H 4.35; N 12.13%; IR (solid ATR), cm^{-1}: 1585, 1615 (NO_2), 2853, 2920 (CH, CH_2), 3320 (OH); ^{1}H-NMR (dmso-d_6+TFA, δ ppm, *J* Hz): 8.84(d, 1H, H-3', 2.4); 8.19(dd, 1H, H-5', 2.4, 9.9); 7.45(d, 1H, H-6', 9.9); 5.13(d, 0.85*H, H-1α, 3.5); 4.79(d, 0.15*H, H-1β, 7.5); 3.72-3.28(m, 6H, H-2-3-4-5-6). ^{13}C-NMR (dmso-d6+TFA, δ ppm): 149.50(C-1'); 135.45(C-4'); 130.05(C-2'); 129.80(C-3'); 123.84(C-5'); 117.69(C-6'); 90.90(C-1); 74.11(C-3); 72.89(C-4); 70.64(C-5); 61.42(C-2); 57.95(C-6).

Determination of D-glucosamine (1) from pharmaceutical preparations

Finely ground (5 to 10 mg) preparation was suspended in 2 mL of methanol and stirred at 50˚C for 10 min. The filtered solution was treated with a slight excess of $NaHCO_3$ for neutralizing the acids accompanying the D-glucosamine and/or chondroitin, stirred for 15 min. then treated with two molar equivalents of **2** or **4a** and stirred at 50˚C for another 10 min. (when using **2**) or 30 min. (when using **4a**). One more mL of methanol was added to the reaction mixture. Then 5 μL of this solution was subjected to TLC in one of the following alternatives: (i) analytical silica gel plates RP-18F_{254} (Merck), mobile phase ethanol:water (6:3, v/v), producing one spot; (ii) HPTLC analytical plates CHIR (Merck), mobile phase H_2O:EtOH (9:1, v/v), producing two spots; (iii) analytical silica gel plates GF_{254} (Merck), mobile phase dichloromethane:methanol (8.5:1.5,v/v), producing two spots. The detection was achieved either by UV-Vis at 254 nm, or by fluorescence at 360 nm.

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