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Aracyl Triflates as Derivatizing Agents for Biological Betaines

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Several trifluoromethanesulfonates of general structure $ArCOCH_2OTf$ were prepared and their suitability for use as derivatizing agents for the HPLC analysis of betaines was assessed. Four of them (Ar = 2′-naphthyl, 2′-fluorenyl, 6′-methoxynaphthacyl, and 2′-phenanthrenacyl) showed promise for use with UV and/or fluorescence detectors, with the last potentially the most promising.

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Introduction

Many betaines play important roles in physiology and biochemistry. For example, carnitine and its acyl esters are key intermediates in acyl group transport across the mitochondrial membrane,[1–3] and glycine betaine ($Me_3N^+CH_2CO_2^-$) is involved in cell volume regulation and in the metabolism of methyl groups.[4–6] There is a demand for an accurate and reliable analytical method for compounds of this type. There are several methods that have been used to determine carboxylic acids in biological systems. Mainly they are based on treating the carboxyl function with a reagent bearing a suitable chromophore/fluorophore, followed by high-performance liquid chromatography (HPLC) analysis of the product using UV or fluorescence detection. However, most of these reagents fail to react with the less reactive carboxylate group of a betaine. An ideal derivatizing reagent for the latter would be one that reacted rapidly and quantitatively with betaines, and possessed a strong UV absorbance or fluorescence at a wavelength distinct from that of common HPLC buffers and mobile phases. It would also be an advantage if it could be synthesized in a small number of steps from commercially available starting materials. While a lack of reactivity towards other species likely to be present would also be an advantage, it is not essential, provided the products formed did not interfere with the HPLC analysis.

Of the common reagents employed to analyze carboxylic acids in biological systems, the one that comes closest to fulfilling these requirements is 2-(4′-bromophenyl)-2-oxoethyl trifluoromethanesulfonate (4′-bromophenacyl triflate), and this has been used by several groups.[7–10] Unfortunately, this compound, while stable, easily prepared in two steps from commercially available 4-bromobenzoyl chloride, and capable of reacting rapidly and quantitatively with betaines to give their 4′-bromophenacyl esters, does not fluoresce, and the absorption maxima of its betaine derivatives in the UV region, while fairly high ($\varepsilon \approx 14000$), come at a relatively short wavelength (λ 260 nm). There is an obvious case to be made for seeking an alternative to the 4′-bromophenacyl chromophore that offers superior optical characteristics (absorption at longer wavelengths and/or a higher extinction coefficient), or, even better, is a good fluorophore, as fluorescence detection is normally more sensitive.

The major limiting factor for any potential aracyl chromophore/fluorophore is that it needs to be capable of surviving the conditions required to prepare and to use the reagent. Such a constraint particularly affects fluorophores, as some of the most useful and widely used are chemically quite reactive. There are several synthetic routes to α-ketotriflates such as 4′-bromophenacyl triflate available. The one that appears the simplest and most logical, the reaction of an aracyl halide with silver triflate, fails. Less convenient, but effective, alternatives that have been, or could be, used include the acylation of α-hydroxy ketones by triflic anhydride,[11,12] the oxidation of silyl enol ethers using hypervalent iodine compounds,[13] or the treatment of diazoketones with triflic acid.[12] From the point of view of simplicity and the availability of suitable precursors the last is the method of choice.

The diazoketone intermediate is conveniently prepared by one of two routes (see Scheme 1). Method A is best suited to making small amounts of material, as larger quantities require the preparation and use of diazomethane on a large scale, which, in view of the latter's toxicity, carcinogenicity, and instability, constitutes a potential hazard. The second route (method B) makes use of the Regitz diazo transfer reaction[14] and uses the methyl ketone rather than the acid chloride. The ketonic methyl is subjected to a crossed Claisen condensation with methyl formate to give the sodium salt of the β-keto aldehyde, which in the next step is reacted with *p*-toluenesulfonyl azide to give the diazoketone. While

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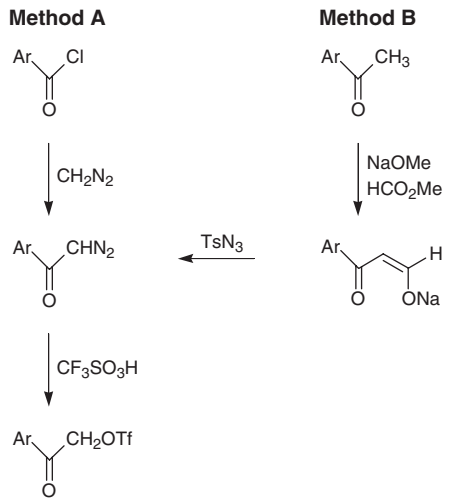
this method is not itself entirely hazard-free (it involves the preparation and use of *p*-toluenesulfonyl azide), it is the one of choice when larger amounts of material are involved. It has the added advantage that *p*-toluenesulfonyl azide, unlike diazomethane, can be stored indefinitely in a freezer until required.

Conversion of diazoketones to α-ketotriflates can be achieved by treatment with triflic acid in liquid sulfur dioxide.[12] Although this method gave acceptable results, dichloromethane at low temperatures proved more convenient and gave equal or superior yields. In our search for suitable chromatophore/fluorophores, we limited ourselves mostly to those that already contained either an acetyl or a carboxyl functional group and which were commercially available. Good fluorophores normally also absorb strongly in the UV region, so our selections were oriented towards these. The most successful turned out to be ones based on fused carbocyclic ring systems.



Scheme 1.

Results and Discussion

The following triflates were selected for screening as potential candidates for derivatizing agents: 4′-methoxyphenacyl triflate, 2′-fluorenacyl triflate, 3′,4′,5′-trimethoxyphenacyl triflate, 2′-naphthacyl triflate, 9′-xanthenacyl triflate, 6′-methoxynaphthacyl triflate, 4′-phenoxyphenacyl triflate, and 2′-phenanthrenacyl triflate. Of the eight, 3′,4′,5′-trimethoxyphenacyl triflate and 4′-phenoxyphenacyl triflate decomposed during attempts to purify them, and 4′-methoxyphenacyl triflate, while obtained pure and successfully derivatized representative betaines, had too short a shelf life to justify detailed investigation for possible laboratory use. This left five reagents, namely the 9′-xanthenacyl, 2′-fluorenacyl, 2′-naphthacyl, 6′-methoxynaphthacyl, and 2′-phenanthenacyl triflates, for further investigation. A cursory check revealed that 9′-xanthenacyl triflate did not absorb strongly in the UV region, and fluoresced only weakly. In view of this, and the observation that the yield in the derivatization step was poor, this compound was not investigated further. This left four potential reagents for detailed study. All were used to derivatize a representative betaine, namely glycine betaine. Derivatization involved mixing the betaine and the triflate together in acetonitrile containing a small amount of magnesium hydroxide. The spectroscopic properties of the derivatives obtained were determined, and the relevant data are summarized in Table 1. Also included for comparison purposes are the

Table 1. Extinction coefficients and fluorescence properties of aracyl triflates
Solvents: propan-2-ol/water (75%), acetonitrile/water (90%)

	4′-Bromophenacyl		2′-Naphthacyl		2′-Fluorenacyl		6′-Methoxynaphthacyl		2′-Phenanthrenacyl	
	Pr^iOH/H_2O	MeCN/H_2O	Pr^iOH/H_2O	MeCN/H_2O	Pr^iOH/H_2O	MeCN/H_2O	Pr^iOH/H_2O	MeCN/H_2O	Pr^iOH/H_2O	MeCN/H_2O
Aracyl triflate										
λ_{max} [nm]	260	257	249	249	314	312	241	243	266	267
ε_{max}	14000	15900	35400	39600	17500	25900	26900	30500	49000	58500
λ_{Ex} [nm]			252		231[B]	306[B]	247	260	266	265
λ_{Em} [nm]			418		350[B]	350[B]	431	409	437	418
Relative fluorescence[A]			100	–[C]	282[B]	516[B]	1400	550	1666	733
Glycine betaine derivative										
λ [nm]	260	260	249	249	314	314	241	241	266	266
Peak area (UV)[D]	38	21	100	52	53	31	60	27	97	60
λ_{Ex} [nm]			252	250	335	327	245	260	267	260
λ_{Em} [nm]			431	413	396	379	451	431	463	433
Peak area (fluorescence)[D]			100	1.5	12.5	<0.2	212.5	12.5	178.5	82

[A] Arbitrary units relating to peak height on fluorescence spectrophotometer (for 2′-naphthacyl triflate this value is 100 in 75% propan-2-ol).
[B] These values may be due to an impurity (see text).
[C] None detected.
[D] Area of derivative peak following HPLC separation relative to 2′-naphthacyl triflate derivative (area 100) in 75% propan-2-ol.

corresponding properties of 4′-bromophenacyl triflate and its glycine betaine derivative.

For UV detection, the ideal derivatizing reagent would have a maximum absorption wavelength of greater than 260 nm and an extinction coefficient at least as great as 4′-bromophenacyl triflate. Of the reagents tested, 2′-naphthacyl, 2′-phenanthrenacyl, and 6′-methoxynapthacyl had absorption maxima at wavelengths close to or shorter than 4′-bromophenacyl triflate, but the extinction coefficients were larger. The glycine betaine derivatives of these reagents had greater UV absorbance than the bromophenacyl derivative, so although the wavelengths used for detection did not avoid the interference from the HPLC buffer and solvents, the signal-to-noise ratios were improved. The other reagent, 2′-fluorenacyl triflate, had an extinction coefficient that was only 1.2 times that of 4′-bromophenacyl triflate; however, the absorption maximum (314 nm) was long enough to be distinct from interference from common HPLC buffers and solvents. The potentially most useful reagent is 2′-phenanthreneacyl triflate, as this had a maximum absorbance at 266 nm and an extinction coefficient more than three times that of 4′-bromophenacyl triflate.

All four of the tested reagents successfully derivatized glycine betaine under the reaction conditions used for derivatization with 4′-bromophenacyl triflate. The resulting glycine betaine derivatives had virtually identical absorbance maxima to those of the parent reagents. Extinction coefficients of these derivatives were not determined, but the measured peak areas, detected by UV, are related to these. The derivatives of 4′-fluorenacyl triflate absorbed only slightly less than derivatives of 4′-bromophenacyl triflate, and less than those of 2′-naphthacyl triflate. However, the former would be more useful in practice because of the longer wavelength of their maximum. Detection at 314 nm is less subject to interference than detection at 260 nm, the position of the maximum for 4′-bromophenacyl triflate, and this results in a superior signal-to-noise ratio. As would be expected, the peak areas of the glycine betaine derivatives of 2′-naphthacyl, 6′-methoxynapthacyl, and 2′-phenanthrenacyl triflates were all greater than that for the 4′-bromophenacyl derivative. 6′-Methoxynaphthacyl triflate would be less useful for UV detection than the other reagents, as detection at 241 nm can be subject to a considerable interference from buffer components, and this affects the signal-to-noise ratio. The derivatives of 2′-phenanthrenacyl triflate have a strong absorption maximum at 266 nm. This is very useful for UV detection, as the background at this wavelength is low.

The derivatization conditions used were ones optimized for 2′-naphthacyl triflate and are not necessarily optimal for the other reagents. This may explain the less than expected sensitivity increase for the 2′-phenanthrenacyl and 6′-methoxynaphthacyl compounds. Larger peak areas could be expected if the derivatization conditions used were optimized for each reagent.

The sensitivity of the assay could be increased considerably if fluorescence detection was possible. All of the reagents in Table 1 had some fluorescence. However, the carbonyl of the $COCH_2OTf$ group both diminishes the intensity of the fluorescence of the aromatic system and decreases the separation of the excitation and emission wavelengths. 2′-Naphthacyl, 6′-methoxynapthacyl, and 2′-phenanthrenacyl triflates had excitation and emission wavelengths considerably separated which is desirable for a derivatizing reagent to be used with fluorescence detection. As the bandwidth of fluorescent detectors in most HPLC instruments is approximately 10–15 nm, well separated wavelengths lead to decreased background interference due to scattered light, and hence to an improved signal-to-noise ratio. However, in the case of the 2′-fluorenacyl triflate, the excitation and emission wavelengths are close enough to cause problems.

The fluorescence properties of the glycine betaine derivatives were similar to those of the derivatizing reagent used, and significant fluorescence was observed when the excitation and emission wavelengths of the reagents were used for their detection. However, in solution, differences in the solvation of the derivatives and the reagents can affect the positions of the fluorescence maxima, so the wavelengths used for detection were optimized. It was found that, in general, the excitation maxima of the glycine betaine derivatives lay close to those of the reagents, while those for emission shifted to a longer wavelength. The wavelengths of the 2′-fluorenacyl triflate and its betaine derivative were not related and the strength of the fluorescence of the former was much stronger than that of the latter. This discrepancy suggests that the reagent may contain a strongly fluorescent impurity. If this impurity did not derivatize glycine betaine, the wavelengths recorded would be the true maxima of the fluorenacyl derivative.

6′-Methoxynaphthacyl triflate and 2′-phenanthrenacyl triflate had greater fluorescence than 2′-naphthacyl triflate, and this was also true of their betaine derivatives. However, the extent of the increased fluorescence was dependent on the solvent used. This means that choice of solvent becomes potentially an important consideration when designing an HPLC system. It was found that for all the reagents less fluorescence was observed in acetonitrile, to the extent that it was negligible for 2′-naphthacyl triflate in that solvent. A considerable decrease was also noted for its derivatives. As the HPLC system is based on an acetonitrile mobile phase, this made 2′-naphthacyl triflate unsuitable as a reagent for fluorescence-based detection.[15,16] The reagents were more strongly fluorescent in propan-2-ol; similar behaviour was noted for the derivatives. Using a propan-2-ol mobile phase, the derivative of 2′-phenanthrenacyl triflate was twice as fluorescent as the same derivative analyzed with an acetonitrile one. Changing the solvent affected the fluorescence of the derivatives to varied extents. In acetonitrile, the 2′-phenanthrenacyl derivative was almost 50 times more fluorescent than the derivatives of the other reagents, but in propan-2-ol the increase is only twofold. The emission maxima of the reagents and the derivatives changed with solvent. Tamaki has shown that the effect of protic solvents on the fluorescence spectra of 2′-acylanthracenes arises from a combination of the non-specific dipolar and the specific hydrogen-bond interactions.[17,18] The decrease in dielectric constant from acetonitrile (38.8) to propan-2-ol (18.3)

decreases the Stokes shift and results in a longer emission maximum. Hydrogen-bond formation in propan-2-ol enhances this shift, but interaction is less in acetonitrile. The absorbance of the derivatives was only changed to a small extent by the solvent.

Conclusions

All four of the potential reagents that were synthesized and investigated in detail represented an improvement on the widely used 4′-bromophenacyl triflate.[7–10,19] All could be readily prepared from commercially available starting materials. Each had a chromophore/fluorophore with a stronger extinction coefficient and fluorescence than 4′-bromophenacyl, and all derivatized betaines rapidly and quantitatively. Of the four, 2′-phenanthrenacyl triflate shows the most promise as a derivatizing reagent for both UV and fluorescence detection in biological samples. The other reagents studied are also useful, as having a range of reagents to choose from increases the range of buffers and solvents that can be utilized. A range of fluorescent reagents is also advantageous for capillary electrophoresis coupled with fluorescent detection, as the reagent can be chosen that best suits the fixed extinction and emission wavelengths available.

Experimental

The carboxylic acid chlorides required for method A were either commercially available or were prepared from the corresponding acids by treating them with either thionyl chloride or oxalyl chloride. The methyl ketones required for method B were all commercially available. ^{1}H and ^{13}C NMR spectra were obtained on a 300 MHz Varian Unity or 500 MHz Varian Inova spectrometer.

Synthesis of the Diazo Ketones

The two methods used for the preparation of the diazo ketones are illustrated below for the case of 2′-diazoacetylnaphthalene.

Method A[20]

A solution of 2-naphthoyl chloride (11 g, 60 mmol) in anhydrous ether (20 mL) was added dropwise over 20 min to a stirred solution of approximately 0.1 moles of diazomethane in ether (300 mL) cooled to 0°C in an ice bath. Stirring was continued for 3 h, and the mixture was then allowed to stand unstoppered in a fume hood overnight to allow evaporation of excess diazomethane. Evaporation of the solution gave a product that was sometimes dark. This could be removed by dissolving it in warm benzene and filtering it through a short (15 cm) silica column. The major impurities were small amounts of methyl 2-naphthoate and 2-chloroacetylnaphthalene. Crystallization of the material from petroleum ether containing a small amount of dichloromethane gave a sufficiently pure product for the next step. The yield of diazo ketone was usually 10–11 g (80–90%).

Other diazo ketones prepared by this route were 4′-methoxydiazoacetophenone (86%), 3′,4′,5′-trimethoxydiazoacetophenone (85%), 9′-diazoacetylxanthene (93%), and 2′-diazoacetylfluorene (81%).

Method B[14]

A well stirred slurry of sodium methoxide (6 g, 110 mmol) in anhydrous ether (80 mL) was cooled in an ice bath and to this was added, dropwise over approximately 15 min, a solution of 2-acetonaphthone (17 g, 100 mmol) and methyl formate (6.6 g, 110 mmol) in sufficient ether to dissolve the former (approximately 50 mL). Stirring was continued for approximately 15 h at room temperature. During this period, the suspension of the sodium methoxide was replaced by one of the sodium salt of the β-keto aldehyde. The precipitate was filtered, washed with anhydrous ether, and allowed to air-dry. The yield (about 22 g) was approximately quantitative, and the product could be used without further purification. If not used immediately, it could be stored until needed.

The sodium salt of the β-keto aldehyde (22 g, 100 mmol) was suspended in ethanol (250 mL) and cooled to 0–5°C in an ice bath. To this was added dropwise, with stirring, *p*-toluenesulfonyl azide[21] (20 g, 100 mmol). (The rate of addition was not critical, as heat did not appear to be evolved.) The ice bath was removed, and stirring continued at room temperature for 4 h. During this time the suspension turned yellow. The ethanol was then evaporated at 30°C under reduced pressure, approximately 250 mL of ether and 200 mL of 10% sodium hydroxide was added to the residue, and the contents of the flask were shaken until any solid present had dissolved in the ether layer. This layer was then separated, washed with water, and dried. Evaporation of the ether gave the diazo ketone as a yellow solid. This material was sufficiently pure for conversion into the triflate. The yield was approximately 18 g (92%).

Diazo ketones prepared by this route were 4′-phenoxydiazoacetophenone (54%), 2′-diazoacetylfluorene (50%), 6′-methoxy-2′-diazoacetylnaphthalene (93%), and 2′-diazoacetylphenanthrene (85%).

In the case of the last two diazo ketones, the method was modified slightly because of the low solubility of both the methyl ketone starting material and the products in the solvents used. For the 6′-methoxy-2′-diazoacetylnaphthalene compound, it proved necessary to use about three times as much ether in the Claisen step, and in the extraction of the diazo compound dichloromethane was used rather than ether. For the 2′-phenanthrenyl compound, the starting material and products were even less soluble. The condensation step was allowed to continue for 2 days, and the diazo transfer step for 3 days. The diazo ketone product was then isolated by multiple extractions of the reaction mixture with dichloromethane.

Conversion of the Diazoketones into α-Ketotriflates

The diazo ketones prepared by method A or B were converted into α-keto triflates by treatment with triflic acid, either by the method of Vedejs[12] or by treatment with triflic acid in dichloromethane. The main difference between the two methods is the different solvent used. The first uses liquid SO_2 at −78°C and the second, dichloromethane. The results were comparable, but the second method was more convenient.[22] The procedure for the CH_2Cl_2 method is given below.

Dichloromethane (30 mL) was placed in a 100-mL conical flask. The flask and its contents were cooled to about −20°C by means of a dry ice/ethylene glycol bath, and triflic acid (4.5 mL, 50 mmol) was added slowly to the stirred solution. A solution of the diazo ketone (5 g, 25 mmol) dissolved in approximately 20 mL of dichloromethane was then added dropwise over 10–15 min to the well stirred solution. The solution initially turned orange and then darkened. After the addition was complete, stirring under nitrogen was continued for about 1 h at −20°C. The mixture was then allowed to warm to room temperature and stand overnight.

Excess triflic acid was neutralized by the cautious addition of aqueous sodium bicarbonate (10 mL) to the stirred solution. The mixture was then poured into a 250-mL separating funnel, washed several times with aqueous sodium bicarbonate, and then with water. The dark dichloromethane layer was separated, dried with anhydrous sodium sulfate, and the dichloromethane evaporated to give the triflate as a dark, but normally crystalline, product. At this point the yield was usually quite high, and normally a single crystallization gave a product sufficiently pure for subsequent use. Purer products could be obtained by further recrystallization of this material, but this often led to considerable losses.

Details for individual compounds are given below. Unless otherwise stated the conversion was carried out in dichloromethane.

2′-Naphthacyl Triflate

Crystallization of the crude product from benzene/petroleum ether gave colourless *leaflets*, mp 109–110°C, in approximately 30% yield (Found: C 49.0, H 3.1. $C_{13}H_9F_3O_4S$ requires C 49.1, H 2.9%). δ_H (300 MHz, $CDCl_3$ and Me_4Si) 8.35 (1 H, s, ArH), 7.88–7.97 (4 H, m, ArH), 7.56–7.68 (2 H, m, ArH), 5.77 (2 H, s, CH_2).

2′-Fluorenacyl Triflate

Crystallization from dichloromethane/pentane gave light purple *flakes* (30%), mp 140–142°C (Found: C 53.8, H 3.2. $C_{16}H_{11}F_3O_4S$ requires C 53.9, H 3.1%). δ_H (300 MHz, $CDCl_3$ and Me_4Si) 8.08 (1 H, s, ArH), 7.86–7.91 (3 H, m, ArH), 7.59–7.63 (1 H, m, ArH), 7.42–7.46 (2 H, m, ArH), 5.71 (2 H, s, CH_2), 3.99 (2 H, s, H_29').

6′-Methoxy-2′-naphthacyl Triflate

This product was extremely difficult to free from coloured impurities. Crystallization from benzene/petroleum ether gave white *flakes* with a greenish tinge, mp 132–134°C, in approximately 30% yield. More strongly coloured fractions obtained (ranging up to dark green) appeared substantially pure by ^1H NMR spectroscopy (Found: C 55.7, H 2.9. $C_{15}H_{13}F_3O_6S$ requires C 55.4, H 3.0%). δ_H (300, MHz, $CDCl_3$ and Me_4Si) 8.28 (1 H, s, ArH), 7.91 (1 H, d, *J* 8.8, ArH), 7.81–7.86 (2 H, m, ArH), 7.24 (1 H, d, *J* 8.8, ArH), 7.17 (1 H, s, ArH), 5.76 (2 H, s, CH_2), 3.96 (3 H, s, OCH_3).

2′-Phenanthrenacyl Triflate

Because of the low solubility of the diazo ketone in dichloromethane, the diazo ketone was added as a slurry. This appeared to have no effect on the yield. The crude product was substantially pure by NMR spectroscopy, and formed in close to quantitative yield. Recrystallization from dichloromethane gave light brown *crystals*, mp 120–122°C (Found: C 55.7, H 2.9. $C_{17}H_{11}F_3O_6S$ requires C 55.4, H 3.0%). δ_H (300 MHz, $CDCl_3$ and Me_4Si) 8.79 (1 H, d, *J* 8.8, ArH), 8.71 (1 H, d, *J* 7.3, ArH), 8.39 (1 H, s, ArH), 8.12 (1 H, d, *J* 8.8, ArH), 7.94 (1 H, d, *J* 6.8, ArH), 7.86 (1 H, d, *J* 8.8, ArH), 7.81 (1 H, d, *J* 8.8, ArH), 7.71–7.74 (2 H, m, ArH), 5.82 (2 H, s, CH_2).

9′-Xanthenacyl Triflate

The crude product was obtained in approximately 90% yield using Vedejs' method.[12] Recrystallization from pentane/dichloromethane gave white fibrous *crystals*, mp 98–100°C, in low yield (Found: C 51.8, H 3.0. $C_{16}H_{11}F_3O_4S$ requires C 51.6, H 3.0%). δ_H (300 MHz, $CDCl_3$ in Me_4Si) 7.34–7.41 (2 H, m, ArH), 7.10–7.26 (6 H, m, ArH), 5.15 (1 H, s, H9′), 4.83 (2 H, s, CH_2).

4′-Methoxyphenacyl Triflate

The crude product, also obtained using Vedejs' method,[12] crystallized from dichloromethane/pentane as fine white *needles* (49%), mp 73–74°C (Found: C 40.5, H, 3.2. $C_{10}H_9F_3O_5S$ requires C 40.3, H 3.0%). δ_H (300 MHz, $CDCl_3$) 7.84 (2 H, d, *J* 9.0, ArH), 6.96 (2 H, d, *J* 9.0, ArH), 5.58 (2 H, s, OCH_2), 3.87 (3 H, s, OCH_3). This compound decomposed over a few weeks.

4′-Phenoxyphenacyl Triflate

All attempts to purify the crude product led to its rapid decomposition.

Spectrophotometric Measurements

Ultraviolet adsorption data were recorded on a Philips PU 8730 UV-visible spectrophotometer. Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer. A quartz (10-mm square) sample cell was used and the slit width for both excitation and emission was 5 nm. Standard solutions (10 μM) were prepared in 75% propan-2-ol, 25% water, or 90% acetonitrile, 10% water.

For the formation of all derivatives, an aqueous standard containing glycine betaine was used. The final concentration of sample injected into the HPLC port after derivatization was 7 μM. The procedure used was essentially the same as that used previously by us for the derivatization of betaines using *p*-bromophenacyl triflate.

To 50 μL of standard in acetonitrile (250 μL) in an eppendorf tube was added derivatizing reagent (50 μL, 10 mM) in acetonitrile and $Mg(OH)_2$ (5 μL, 0.1 g L^{-1} suspension of MgO in water). This mixture was vortexed briefly and shaken for 5 min, before being centrifuged for 5 min at 11600*g*. Samples were removed immediately from centrifuge and 200 μL of supernatant was transferred to an HPLC vial, and the samples were diluted with acetonitrile (200 μL). Vials were capped tightly to avoid evaporation of sample and 10 μL of sample was injected onto the column.

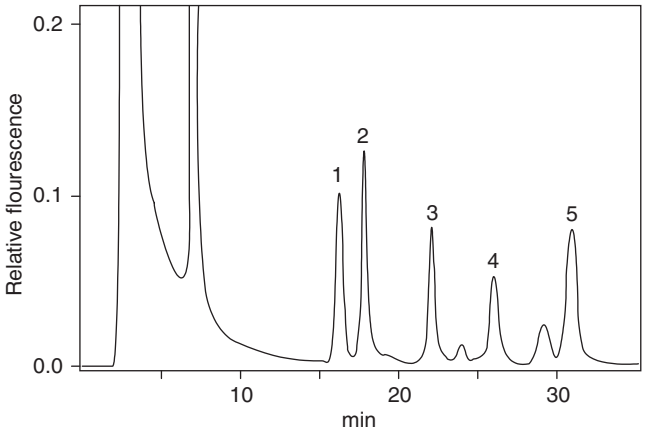


Fig. 1. HPLC trace of standards of natural betaines derivatized with 2′-phenanthrenacyl triflate. [Peak 1, acetylcarnitine ($Me_3N^+CH_2CH(OAc)CH_2CO_2^-$); peak 2, arsenobetaine ($Me_3As^+CH_2CO_2^-$); peak 3, glycine betaine; peak 4, dimethylsulfoniopropionate ($Me_2S^+CH_2CH_2CO_2^-$); peak 5, carnitine ($Me_3N^+CH_2CH(OH)CH_2CO_2^-$).]

HPLC was performed on a Shidmadzu 10 AVP. The maximum ultraviolet absorption of derivatives was determined using data gathered during analysis of standards. Fluorescence data were obtained by collecting fractions containing glycine betaine derivatives after HPLC.

HPLC analyses used as the mobile phase either 75% propan-2-ol/water with 1.25 mmol L^{-1} succinic acid, 0.625 mmol L^{-1} triethylamine eluting buffer, or 90% acetonitrile/water with 7 mmol L^{-1} glycolate eluting buffer. Columns were either a Brownlee 5-μm silica 100 by 4.6 mm cartridge or a Phenosphere (SCX 80A 5 μM, 250 by 4.6 mm) with a guard cartridge of the same packing. Columns were maintained at 40°C during analyses.

A chromatogram obtained from an analysis using a Phenosphere column and 90% acetonitrile/glycolate eluent of the product obtained following derivatization of a standard mixture of natural betaines by 2′-phenanthrenacyl triflate is given in Fig. 1. (The two unidentified peaks were also present in a blank.)

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