Biosynthesis of tetraponerine-8, a defence alkaloid of the ant *Tetraponera* sp.[1]

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This paper is dedicated to Professors David B. MacLean and Ian Spenser

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The administration of sodium [1-^{14}C] and [2-^{14}C] acetate, L-[U-^{14}C] glutamic acid, γ-amino [U-^{14}C] butyric acid, L-[U-^{14}C] ornithine hydrochloride, and [1,4-^{14}C] putrescine dihydrochloride to *Tetraponera* sp. ants resulted in the formation, for each feeding experiment, of labelled tetraponerine-8 (**2**). The distribution of radioactivity into **2** was established by chemical degradation. The results obtained favor the hypothesis that the pyrrolidine ring of tetraponerine-8 is derived from L-glutamic acid via L-ornithine and putrescine, while the 12-carbon chain is derived from the combination of six acetate units.

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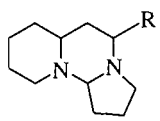
L'administration de [1-^{14}C] et [2-^{14}C] acétate de sodium, d'acide L-[U-^{14}C] glutamique, d'acide γ-amino [U-^{14}C] butyrique, de L-[U-^{14}C] ornithine·HCl et de [1,4-^{14}C] putrescine·2HCl à des fourmis de l'espèce *Tetraponera* sp. conduit à l'isolement de tétraponérine-8 radioactive. La distribution de l'activité au sein de la molécule a été déterminée par dégradation chimique. Les résultats obtenus confortent l'hypothèse que le cycle pyrrolidine de la tétraponérine-8 se construit à partir de l'acide L-glutamique via la L-ornithine et la putrescine. La chaîne à 12 atomes de carbone se formerait quant à elle par condensation de six unités acétate.

Introduction

The tetraponerines are a group of tricyclic alkaloids isolated from the venom of the Neo Guinean ant *Tetraponera* sp. (1, 2). These insecticidal substances are 5-alkyldecahydro-pyrido-[1,2-*c*]pyrrolo[1′,2′-*a*] pyrimidines (**1**)[4] differing from each other by the length of the alkyl chain and (or) by the configuration of the cyclic junctions. Such a skeleton is new among natural products. The biosynthetic pathway to the tetraponerines is therefore a matter of particular interest.



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The aim of the present paper is to shed light on the biosynthetic pathway of the tetraponerine ring system as represented by tetraponerine-8 (**2**), the major constituent of the ant's venom. To this end, we have developed a degradation procedure for **2**, which would allow us to locate radioactivity after administration of ^{14}C-labelled compounds to *Tetraponera* sp. workers to test the likelihood of hypothetical biosynthetic pathways.

Results and discussion

First of all, we needed adequate supplies of **2** to develop a degradation scheme of this alkaloid as well as to serve as carrier for labelled samples of **2** obtained from each feeding experiment. This was achieved by preparing (±)-tetraponerine-8, utilizing the stereoselective total synthesis we recently reported (3, 4).

To evaluate the distribution of label in **2**, the sequence of reactions shown in Fig. 1 was used. The N17—C5 bond[5] could be cleaved selectively by catalytic hydrogenation in the presence of hydrochloric acid. Treatment of the resulting diamine **3** with methanesulfonyl chloride furnished the sulfonamide **4**. Pyrolysis of the tetraalkylammonium hydroxide **5**, obtained by quaternization of **4** with methyl iodide followed by conversion into hydroxide using an anion exchange resin, led to a mixture from which olefin **6** (27–46%) and *N*-methylpyrrolidine (**7**; 25–47%) could be isolated. Interestingly, of the four possible isomeric olefins that might theoretically result from the elimination of *N*-methylpyrrolidine, only isomer **6** (E Δ^9) was obtained. This suggests that the elimination is stereoselective and proceeds preferentially through a E2 *syn* elimination taking place from a *syn*-periplanar conformation (5, 6). The elimination is also regioselective. The absence of a detectable amount of $\Delta^{9(12)}$ isomers can be explained by the influence of the sulfonamide group that renders the C-10 protons of **5** more acidic than the C-12 protons. The volatile *N*-methylpyrrolidine was trapped in liquid nitrogen and converted into its oxalate. The latter could be recrystallized repeatedly without difficulties whereas the corresponding hydrochloride was too hygroscopic to be purified to constant activity by recrystallization. Olefin **6** was further cleaved by oxidation with the mixture $NaIO_4$–$KMnO_4$ (7) into *N*-mesylpipecolic acid and caproic acid, which were purified by chromatography as their *p*-bromophenacyl esters **8** and **9**, respectively.

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[4]IUPAC numbering.

[5]For convenience, tetraponerine-8 and all its degradation products have been numbered according to ref. 1.

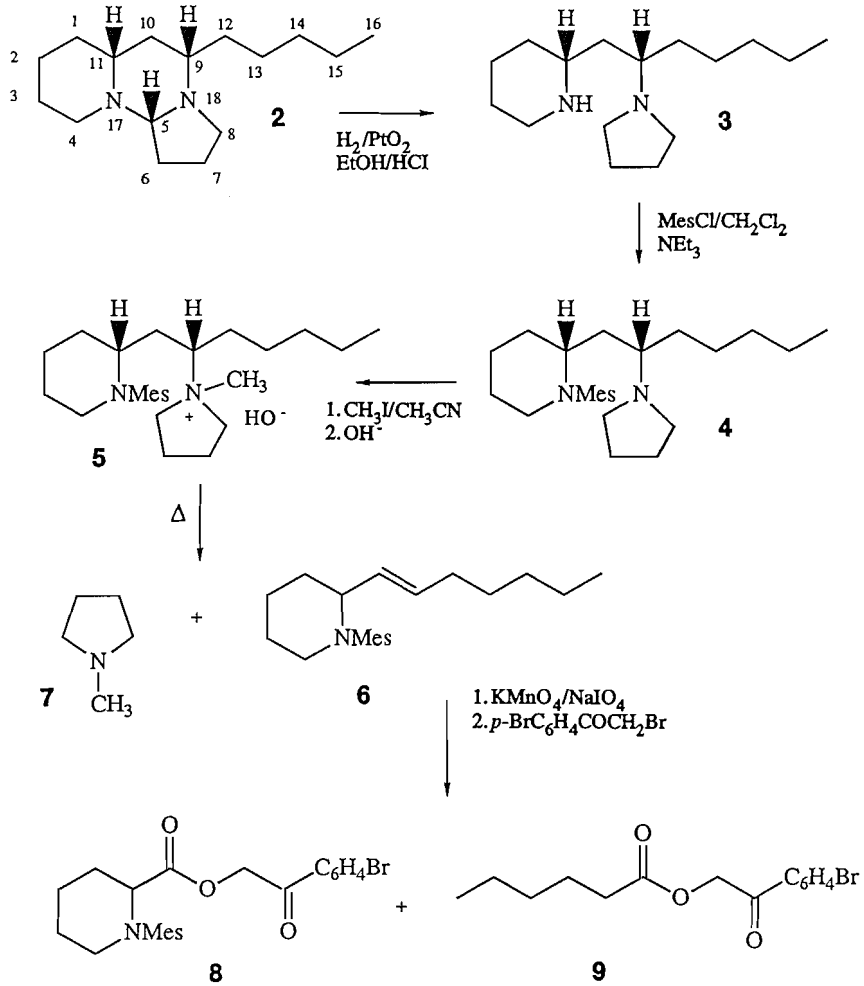

FIG. 1. Degradation of tetraponerine-8.

Insofar as structural relationships can be considered as a guide to biogenesis, the $C_{16}N_2$ skeleton of tetraponerine-8 might be thought to originate by condensation between either a suitably functionalized polyacetate chain such as **10** and putrescine (Fig. 2, pathway A), or a suitably functionalized 2-alkylpiperidine such as **11** and γ-aminobutyric acid (Fig. 2, pathway B). To test these alternative hypotheses, tracer experiments were performed by feeding *Tetraponera* sp. ants with aqueous solutions of sodium [1-^{14}C] and [2-^{14}C] acetate, L-[U-^{14}C] glutamic acid, γ-amino [U-^{14}C] butyric acid, L-[U-^{14}C] ornithine hydrochloride, and [1,4-^{14}C] putrescine dihydrochloride, respectively. A summary of the results of these experiments is presented in Table 1. Each experiment yielded radioactive tetraponerine-8, which was isolated as described in the experimental section. Before applying the degradation scheme of Fig. 1, inactive **2** was added as carrier to each isolated sample. These were then divided in two parts, each of which was separately degraded. After isolation of labelled sulfonamide **4**, inactive **4** was added as carrier and the resulting diluted sample was flash chromatographed repeatedly until constant activity was reached. The relative specific activities measured for the different degradation products are listed in Table 2 for the two replicates.

Sodium [2-^{14}C] acetate proved to be much more efficiently utilized than sodium [1-^{14}C] acetate for the formation of tetraponerine-8. This phenomenon is readily understood when one considers the fate of the carbon atoms of acetic acid when it enters the tricarboxylic acid cycle (8). The samples of tetraponerine-8 derived from the [1-^{14}C] and [2-^{14}C] acetate feeding experiments yielded olefin **6** (corresponding to carbon atoms C-1 to C-4 and C-9 to C-16) and *N*-methylpyrrolidine (**7**, corresponding to carbon atoms C-5 to C-8), which contained about 2/5 and 3/5 of the activity of intact tetraponerine-8, respectively. This suggests that acetate enters into both parts of the molecule by two independent pathways, its incorporation into **7** being more efficient than into olefin **6**. Moreover, within experimental error, the activity of olefin **6** is equally divided between the pipecolic acid moiety (corresponding to carbon atoms C-1 to C-4 and C-10 and C-11) and the caproic acid moiety (corresponding to carbon atoms C-12 to C-16 and C-9), suggesting that the 12-carbon linear chain of tetraponerine-8 might be formed by a linear combination of acetate units.

The results of feeding experiment 3 showed that the pattern of incorporation of activity from L-[U-^{14}C] glutamic acid into the tetraponerine skeleton was similar to that of acetate except that the incorporation into the pyrrolidine moiety was still more efficient than into olefin **6**. This could be explained by admitting that L-glutamic acid is an intermediate between acetate and the pyrrolidine ring moiety of tetraponerine-8 and that this precursor, after having been catabolized into acetate, is also incorporated into the 12-carbon linear chain of tetraponerine-8. It is well known that the two-carbon fragment at the oxidation level

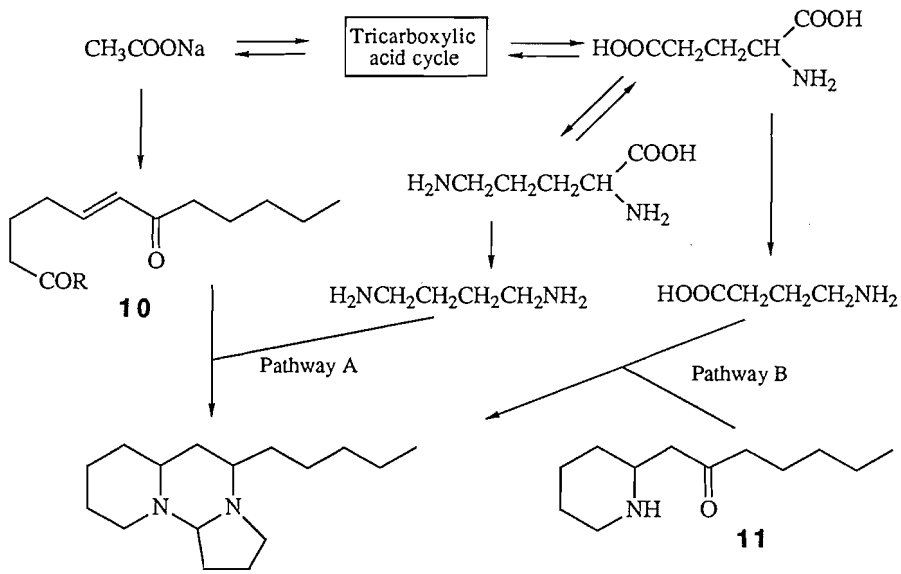

FIG. 2. Hypothetical biosynthetic scheme of tetraponerine-8.

TABLE 1. Incorporation of labelled compounds into tetraponerine-8

Experiment n°	Compound administered	Total activity administered (μCi)	Number of ants fed	Isolated amount of **2** (mg)	Specific activity of **2** (μCi/μmol)	Incorporation (%)
1	Na[1-^{14}C] acetate	150	850	2.0	9.32×10^{-3}	0.05
2	Na[1-^{14}C] acetate	50	400	4.3	8.88×10^{-3}	0.31
3	L-[U-^{14}C] Glutamic acid	50	780	1.7	9.68×10^{-3}	0.13
4	γ-amino[U-^{14}C] butyric acid	50	1150	5.2	3.50×10^{-3}	0.14
5	L-[U-^{14}C] Ornithine HCl	50	1560	5.3	3.09×10^{-2}	1.31
6	[1,4-^{14}C] Putrescine·2HCl	50	1700	6.4	3.95×10^{-2}	2.02

TABLE 2. Observed distribution of label within the degradation products of **2** (each experiment has been duplicated)

Experiment n°	**6** RSA[a]	**7** RSA[a]	**8** RSA[b]	**9** RSA[b]
1	35/34	54/54	52/33	44/36
2	44/42	55/50	43/44	54/56
3	27/24	77/83	64/40	45/74
4	48/45	55/50	45/48	46/49
5	14/16	81/79	64/58	61/55
6	0/0	87/105	—	—

[a]Relative specific activity (%; sulfonamide **4** = 100).
[b]Relative specific activity (%; olefin **6** = 100).

of acetate is derivable from glutamic acid by degradation via α-ketoglutarate and the tricarboxylic acid cycle (9). Moreover, according to classical biogenetic concepts, the pyrrolidine ring of a variety of alkaloids is derived from L-ornithine, which is metabolically interconvertible with L-glutamic acid (10, 11).

Thus, the observed mode of incorporation of these three precursors into tetraponerine-8 is consistent with the hypotheses outlined in Fig. 2. At this stage of the work it was not yet possible to distinguish between the two alternative pathways of incorporation of L-glutamic acid into tetraponerine-8. This demanded additional feeding experiments with γ-amino [U-^{14}C] butyric acid, L-[U-^{14}C] ornithine hydrochloride, and [1,4-^{14}C] putrescine dihydrochloride.

The labelling pattern that was observed after incorporation of γ-aminobutyric acid (experiment 4) was almost identical to that obtained with acetate. This indicates that this acid does not serve as a true intermediate but suffers degradation to acetate. Indeed, γ-aminobutyric acid could enter the tricarboxylic acid ring via succinic semialdehyde and succinic acid (11). Conversely, the results of feeding experiments 5 and 6 were particularly informative. Indeed, L-ornithine and putrescine not only entered the tetraponerine skeleton with high incorporation, and putrescine better than L-ornithine, but the pyrrolidine moiety also contained most of the labelling of intact tetraponerine-8. Such a distribution of the activity led to the inference that these two metabolites serve as specific precursors for the pyrrolidine ring of tetraponerine-8.

It is well established that, in plants, L-ornithine and L-glutamic acid are readily interconverted reversibly while the formation of putrescine from L-ornithine by the action of the enzyme ornithine decarboxylase is an irreversible process (11).

Such pathways seem also to occur in the poison gland of *Tetraponera* sp. since, on incorporation of labelled L-ornithine or L-glutamic acid, some residual activity (15% and 25%, respectively) was recovered in the 12-carbon linear chain, indicating that a certain amount of these amino acids had been catabolized to acetate via the tricarboxylic acid cycle. On the other hand, no residual label could be detected in the carbon atoms of the 12-carbon linear chain after feeding labelled putrescine. All these results support pathway A of the biosynthetic scheme depicted in Fig. 2.

Nothing is known about the precise structure of the 12-carbon intermediate that combines with putrescine to furnish tetraponerine-8. It might be an acid (**10**, R = OH) or an aldehyde (**10**, R = H) similar to **10**. The latter can be regarded either as an oxidized derivative of lauric acid or as a degradation product resulting from the cleavage of, for example, an 18-carbon polyunsaturated fatty acid. Such processes of fracture are well documented and are known to proceed through cleavage of peroxidic fatty acids by a lyase to form aldehydes (11, 12).

In the plant kingdom, the couple ornithine/putrescine is the precursor of the atoms of the pyrrolidine ring of many alkaloids, e.g., the pyrrolizidine alkaloids, nicotine, hygrine, and some tropane alkaloids (10, 11, 13). The results we have presented here demonstrate that such a biogenetic pathway also takes place in the ant *Tetraponera* sp. It is the first time that this has been evidenced in the animal kingdom. Further feeding experiments are in progress to refine the biosynthetic scheme depicted in Fig. 2 (pathway A) and to investigate the biosynthesis of the other tetraponerines present in the defence secretion.

Experimental

General methods

The ^1H and ^{13}C nmr spectra were recorded in $CDCl_3$ at 250 and 62.8 MHz, respectively, using a Bruker WM 250 spectrometer. Chemical shifts are quoted in δ values downfield from internal TMS. Data are reported as follows: chemical shift (multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad), coupling constants in hertz, integration, assignment). The ir spectra were taken with a Bruker IFS 25 instrument. Electron impact mass spectra were recorded on a VG Micromass 7070 F spectrometer. Gas chromatographic (gc) analyses were carried out on a Varian 3700 apparatus using a PDMS capillary column (25 m) and tlc on neutral aluminium oxide $60F_{254}$ (type E; Merck) or silica gel Sil G/UV_{254} (0.25 mm; Macherey–Nagel) precoated plates. Silica gel column chromatographies were performed on silica gel 60 columns (0.04–0.063 mm; Macherey–Nagel) by the flash technique (14) or on Lobar pre-packed columns (size A (240-10); Lichroprep Si 60 (0.04–0.063 mm)). Alumina chromatographies were carried out on neutral aluminium oxide columns (Macherey–Nagel). Radioactive compounds were assayed in a Packard Tri-Carb 1600 TR liquid scintillation analyser using as solvent MeOH (100 μL) with Packard Insta-Gel liquid scintillation cocktail (10 mL). Triplicate samples of each compound were counted under comparable conditions of quenching.

Incorporation experiments

Tetraponera sp. ants (about 1000 per batch) were collected near Bogia along the North coast of Papua New Guinea. Hollow twigs housing ants were kept in 50 × 35 cm plastic trays. Water was provided ad libitum. After 3 days of starvation, the ants were fed with 1 mL of an aqueous solution of sucrose (1 M) containing the labelled precursor. They were killed 10 days later by dropping them into vials containing methanol. The extraction of the alkaloids and the purification of tetraponerine-8 (**2**) were performed as described in ref. 2. The results obtained from the different feeding experiments are reported in Table 1.

Degradation of tetraponerine-8

Each labelled sample of tetraponerine-8 was degraded in duplicate. Results of both replications are presented in Table 2.

Conversion of tetraponerine-8 (2) into sulfonamide 4

Typically, tetraponerine-8 (14.9 mg) was dissolved in ethanol (15 mL), HCl (12.5 N, 0.2 mL) and PtO_2 (7.5 mg) were added, and the mixture was stirred during 1 h under an atmosphere of hydrogen. The catalyst was filtered off and the solution evaporated to dryness in vacuo. The solid residue was dissolved in CH_2Cl_2 and the resulting organic phase was washed with an aqueous solution of NaOH (2 N) and evaporated to dryness. The resulting oil was dissolved in dry CH_2Cl_2 (7 mL), triethylamine (158 μL) and mesyl chloride (56 μL) were added at –78°C, and the solution was then allowed to warm up to room temperature. After evaporation of the solvent the residue was dissolved in CH_2Cl_2 (10 mL), and the organic phase was washed with an aqueous solution of ammonia (1.25%) and evaporated to dryness in vacuo. The resulting yellowish oil was then chromatographed over alumina (eluent: CH_2Cl_2–AcOEt 8:2). This yielded pure sulfonamide **4** (10.9 mg, yield: 55%). Spectral properties of **4**: ir (NaCl film): 2860 and 2796 (Bohlmann bands), 1326 and 1146 (SO_2 asymmetric and symmetric stretchings) cm^{-1}; ^1H nmr: 0.89 (t, 7, 3H, H_3C-16), 1.3–1.8 (m, 16H, CH_2), 1.95 (br s, 4H, H_2C-6 + H_2C-7), 2.05 (m, 1H, HC-9), 2.88 (s, 3H, SO_2CH_3), 2.41–2.77 (m, 4H, H_2C-5 + H_2C-8), 3.06 (ddd, 13/13/3, 1H, HaxC-4), 3.70 (brd, 1H, HeqC-4), 4.13 (m, 1H, HC-11); ^{13}C nmr: 14.0 (C-16), 18.6 (C-2), 22.6 (C-15), 23.7 (C-6, C-7), 25.2 and 25.7 (C-3, C-14), 28.4 (C-13), 30.2, 32.0 and 32.3 (C-1, C-10, C-12), 40.7 (C-4), 40.8 (-SO_2CH_3), 49.7 (C-11), 50.5 (C-5 + C-8), 60.9 (C-9); ms *m/z*: 330 ($M^{+\cdot}$, 1%), 259 ($M^{+\cdot} - C_5H_{11}\cdot$, 34), 162 ($M^{+\cdot} - C_{11}H_{22}N\cdot$, 100), 154 ($M^{+\cdot} - C_7H_{14}NO_2S\cdot$, 89), 98 ($C_6H_{12}N^+$, 5), 84 ($C_5H_{10}N^+$, 9), 70 ($C_4H_8N^+$, 5).

Hofmann degradation

Typically, freshly distilled methyl iodide (25 μL) was added to a solution of sulfonamide **4** (9.2 mg) in acetonitrile (0.5 mL) and the mixture stirred at room temperature for 3 h. After evaporation of the solvent, the solid residue was dissolved in MeOH (1 mL) and the solution eluted with water through a strongly basic anion exchanger column (Amberlite IRA-400, OH^-). The fractions containing the tetraalkylammonium hydroxyde **5** (positive Dragendorff and phenolphthalein tests) were combined and evaporated to dryness under vacuum. The residual yellowish oil was then pyrolyzed at 170°C for 3 h. The volatile components were caught in a liquid nitrogen trap, dissolved in acetone, and analyzed by capillary gc (CPWAX 52B, 120°C). The chromatogram showed a major peak whose retention time was identical (coinjection) to that of an authentic sample of *N*-methylpyrrolidine. Oxalic acid (2.5 mg) was then added to the acetone solution. After evaporation of the solvent, the resulting solid was recrystallized from acetone; this furnished *N*-methylpyrrolidine oxalate (2.3 mg, yield: 47%, mp 99–100°C). Flash chromatography of the solid residue of pyrolysis over silica gel (eluent: hexane–AcOEt 8:2) furnished olefin **6** (3 mg, yield: 41%). Spectral properties of **6**: ir (NaCl film): 1334 and 1146 (SO_2 asymmetric and symmetric stretchings) cm^{-1}; ^1H nmr (C_6D_6): 0.88 (t, 7, 3H, HC-16), 1.10–1.60 (m, 12H, CH_2), 1.87 (dt, 6/7, 2H, H_2C-12), 2.31 (s, 3H, SO_2CH_3), 2.56 (ddd, 13/13/2, 1H, HaxC-4), 3.50 (br d, 1H, HeqC-4), 4.52 (m, 1H, HC-11), 5.42 (ddt, 15.5/7/<1, 1H, HC-10), 5.55 (ddt, 15.5/0.5/6, 1H, HC-9); ^{13}C nmr (C_6D_6): 14.0 (C-16), 19.0 (C-2), 22.5 (C-15), 25.6 (C-3), 28.8 (C-13), 31.0, 31.4 and 32.4 (C-1, C-12, C-14), 38.7 (-SO_2CH_3), 41.4 (C-4), 55.0 (C-11), 125.6 (C-9), 134.9 (C-10); ms *m/z*: 259 ($M^{+\cdot}$, 8%), 188 ($M^{+\cdot} - C_5H_{11}\cdot$, 42), 180 ($M^{+\cdot} - CH_3SO_2\cdot$, 96), 162 ($M^{+\cdot} - C_7H_{13}\cdot$, 100), 84 ($C_5H_{10}N^+$, 36).

Lemieux – von Rudloff oxydation of 6

Typically, olefin **6** (2.9 mg) was dissolved in freshly distilled dioxane (3 mL); aqueous solutions of K_2CO_3 (3.8×10^{-2} M, 1 mL), $NaIO_4$ (0.1 M, 1 mL), and $KMnO_4$ (1.7×10^{-3} M, 1 mL) were added and the mixture was stirred at room temperature. The evolution of the reaction was monitored by tlc. After 24 h, 1 mL of each solution was added again. The reaction was complete after 80 h. The mixture was evaporated to dryness and the residue dissolved in CH_2Cl_2 (5 mL). The organic phase was washed with an aqueous solution of HCl (1 N) and evaporated to dryness. The residue was dissolved in MeOH (1 mL) and

the pH adjusted to 8.5 by adding dropwise a methanolic solution of KOH (0.01 N). The resulting solution was again evaporated to dryness and the residue dissolved into freshly distilled acetonitrile; *p*-bromophenacyl bromide (8 mg) and dicyclohexano-18-crown-6 ether (0.6 mg) were added and the mixture was refluxed for 1 h. The residue obtained after evaporation of the solvent was flash chromatographed on a silica gel column (eluent: toluene–AcOEt 99:1). This yielded *p*-bromophenacyl caproate (2.3 mg, yield: 65%) and *p*-bromophenacyl *N*-mesylpipecolate (2.7 mg, yield: 60%) successively. Spectral properties of **8**: ir (NaCl film): 1748 (ester C=O stretching), 1704 (arylketone C=O stretching), 1586 (phenyl C=C stretching), 1326 and 1148 (SO_2 asymmetric and symmetric stretchings) cm^{-1}; ^1H nmr: 1.20–2.00 (m, 5H, CH_2), 2.37 (br d, 1H, HeqC-1), 2.93 (s, 3H, SO_2CH_3), 3.28 (ddd, 12/12/3, 1H, HaxC-4), 3.73 (br d, 1H, HeqC-4), 4.92 (dd, 3.5/<1, 1H, HC-11), 5.29 (d, 16.5, 1H, OCH), 5.47 (d, 16.5, 1H, OCH'), 7.65 and 7.77 (aromatic protons); ms *m/z*: 406/404 ($[M + H]^+$), 326/324 ($M^{+\cdot} - CH_3SO_2\cdot$), 206 ($C_7H_{12}NO_4S^+$), 190 ($C_7H_{12}NO_3S^+$), 185/183 ($C_7H_4OBr^+$), 162 ($C_6H_{12}NO_2S^+$), 157/155 ($C_6H_4Br^+$), 84 ($C_5H_{10}N^+$). Spectral properties of **9**: ir (NaCl film): 1744 (ester C=O stretching), 1700 (arylketone C=O stretching), 1586 (phenyl C=C stretching) cm^{-1}; ^1H nmr: 0.91 (t, 7, 3H, H_3C-16), 1.26 –1.42 (m, 4H, H_2C-15 + H_2C-14), 1.70 (m, 2H, H_2C-13), 2.48 (t, 7.5, 2H, H_2C-12), 5.27 (s, 2H, OCH_2), 7.63 and 7.78 (aromatic protons); ms *m/z*: 314/312 ($M^{+\cdot}$), 216/214 ($M^{+\cdot} - C_6H_{10}O\cdot$), 200/198 ($M^{+\cdot} - C_6H_{10}O_2\cdot$), 185/183 ($C_7H_4OBr^+$), 157/155 ($C_6H_4Br^+$).

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