A Nitro Analogue of Staurosporine and Other Minor Metabolites Produced by a *Streptomyces longisporoflavus* Strain

YANG CAI†, ANDREAS FREDENHAGEN*, PAUL HUG†† and HEINRICH H. PETER

Pharmaceutical Research Department,
Microbial Chemistry and ††Central Function Research,
CIBA-GEIGY Ltd.,
4002 Basle, Switzerland

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From the staurosporine-producing strain *Streptomyces longisporoflavus* R-19 various minor metabolites were isolated: They include a new compound with a nitro function in C-4′ and other metabolites related to staurosporine. The new structures were elucidated by spectroscopic methods, mainly ^{1}H NMR and ^{13}C NMR and by comparison with TAN-1030A. The new compounds inhibited protein kinase C with IC_{50} values in the nanomolar range.

The isoenzyme family of protein kinase C (PKC) plays a key role in signal transduction and cellular regulation[1]. From the observation that the tumor promoting phorbol esters are able to stimulate PKC activity[2], it was concluded that inhibitors of this enzyme could be useful for cancer chemotherapy. PKC inhibitors have been extensively investigated as potential drugs for the treatment of cancer, inflammation and hypertension[3]. Staurosporine isolated from *Str. staurosporeus* as an alkaloidal antibiotic[4] was later found to be the first compound inhibiting PKC in the low nanomolar range[5]. The absolute stereochemistry of staurosporine was determined only very recently[6]. As staurosporine itself lacks selectivity against other kinases, a series of semisynthetic derivatives of staurosporine was synthesized[7] and tested against a range of different kinases. The *N*-benzoyl derivative CGP 41,251 was selected as the most promising compound for further preclinical profiling[7,8].

In our laboratories staurosporine was produced by fermentation of a new strain isolated from a soil sample collected in Ellora, India and taxonomically classified as *Streptomyces longisporoflavus*. Depending on the cultivation conditions this strain produced a varying range of minor components. The isolation of by-products was initiated in order to provide sufficient material for the evaluation of the potency and selectivity of their PKC-inhibiting activity. In addition we were interested to see whether these by-products could provide further hints on the biosynthesis of this metabolite which has not yet been elucidated with the exception of the building blocks of the chromophore[9].

This communication describes the isolation, physicochemical characterization as well as the structures and the inhibitory activity on selected kinases of a first set of by-products isolated from eluate fractions of a large scale preparation of staurosporine.

Materials and Methods

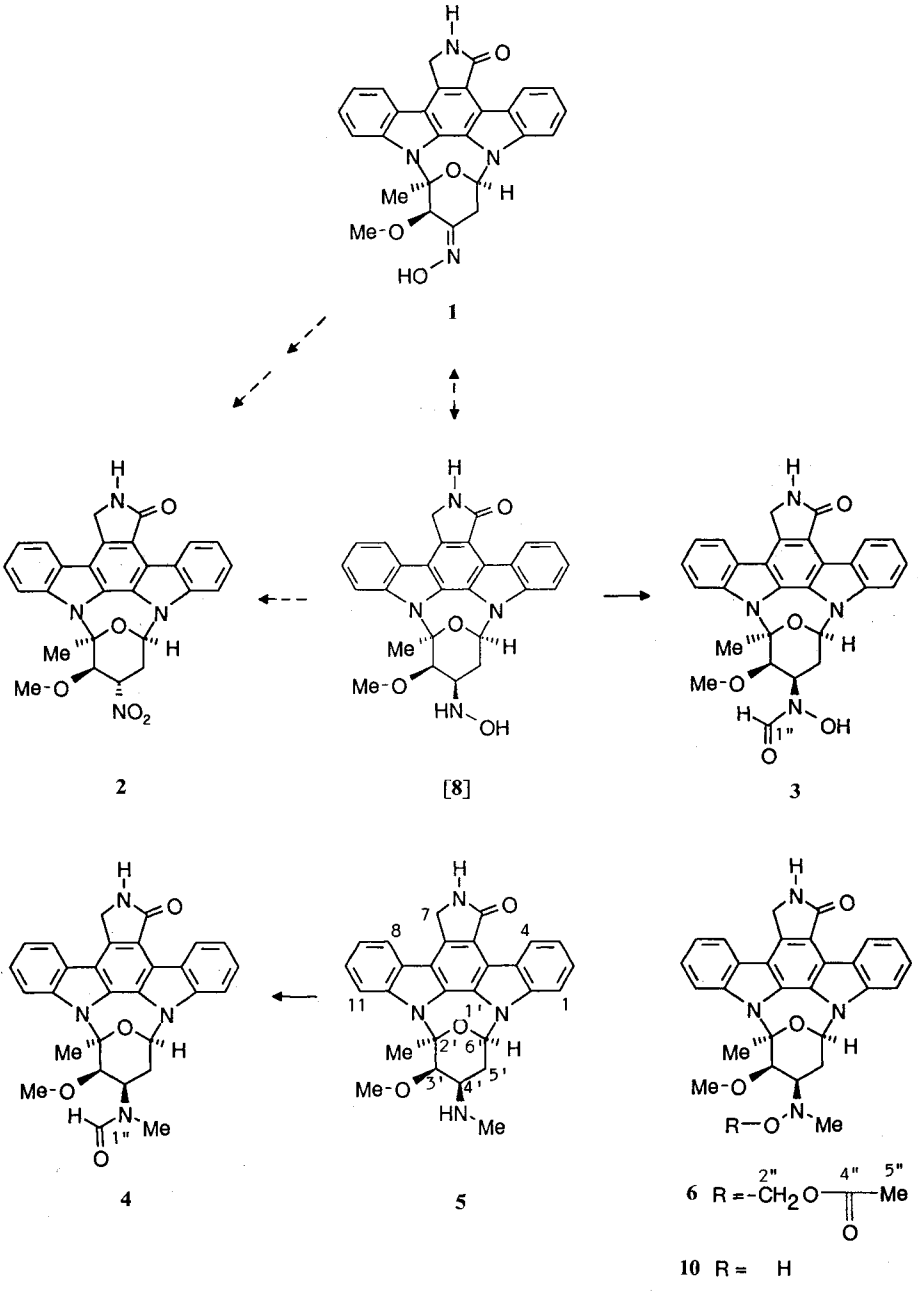
All solvents for silica gel chromatography containing NEt_3 or AcOH were saturated with water. The new *Streptomyces longisporoflavus* R19 strain was cultivated in 2,000 liters fermenters in a medium containing the following ingredients (in g/liter): Noredux starch 60, defatted soya meal 20, $(NH_4)_2SO_4$ 2, $CaCO_3$ 3, antifoam SAG 471 0.5. The sterilized production medium (pH 7.5) was inoculated with 5% of seed cultures grown in the same medium and incubated for 100 hours at 28°C with an aeration rate of 1 liter/liter/minute at a stirring rate of 200 rpm. To the harvest broth a mixture of MeOH-EtOAc, 7 : 5 (v/v; 2,400 liters) was added. Filtration was achieved using a filter aid and the filtrate was concentrated *in vacuo* to 3/4 of its original volume. The concentrate was extracted with 300 liters of $CHCl_3$ and the organic solvent removed. The resulting brown oil was triturated with cyclohexane. Aliquots of the filtered precipitate were applied to a silica gel column and separated into 3 fractions: Fraction 1 eluted with the CH_2Cl_2-2-propanol-acetic acid (95 : 4 : 1) contained mainly unpolar components. The main product staurosporine was eluted in fraction 2 with the solvent mixture CH_2Cl_2-2-propanol-triethylamine (96 : 4 : 0.1). The more polar fraction 3 was eluted with a mixture of the same solvents in the ratio of 90 : 10 : 0.1.

The main metabolite in the unpolar fraction was the oxime analogue TAN-1030A (**1**), which had originally been isolated from cultures of *Streptomyces* sp. C-71799

† Postdoctoral fellow 1989 ~ 1992. Current Address: Beijing Medical University, Building 13-4-601, Beijing 100 083, P. R. China.

Fig. 1. Structures of staurosporine (**5**) and some biosynthetic by-products.

The hypothetical intermediate **8** was not isolated. Compound **10** is the hydrolysis product of **6**.



as an activator of murine macrophages[10]. A slightly more polar elution fraction contained 1.36 g of a by-product which was identified as *N*-formyl-staurosporine **4**, a compound which has already been described in the patent literature[11].

Rechromatography of the unpolar eluate fraction from fraction **1** on silica gel using increasingly polar dichloromethane - acetonitrile - heptane mixtures yielded the two new compounds **2** and **6**. Final purification of **2** (21.6 mg) and **6** (156 mg) was accomplished by rechromatography on reversed phase material (Nucleosil 100-5 C_{18}) using acetonitrile - water, 60 : 40 as eluant. Metabolite **3** (700 mg) was separated from the polar fraction 3 by silica gel chromatography eluting with CH_2Cl_2 - 2-propanol - triethylamine, (95 : 5 : 0.1) and purified by reversed phase chromatography using acetonitrile - water - TFA, (52 : 48 : 0.1) as the eluant. Fractions showing only single peaks in the analytical HPLC were pooled and dried in the high vacuum before performing structural studies.

A considerable number of further minor metabolites will be described in a more detailed forthcoming publication[12].

Results and Discussion

Structure Elucidation

The identification and structure elucidation was mainly based on results from NMR spectroscopy. The ^1H NMR chemical shifts are summarized in Table 1. Results from

Table 1. ^{1}H NMR data chemical shifts of compounds **1**, **2**, **3**, **4**, **6** and **10**.

	1[e]	2[a]	3[b]	4[c]	6[d]	10[a,f]
1	7.56 d	7.68 d	7.60 d	7.54 d	7.60 d	7.57 d
2	7.45 t	7.49 t	7.50 t	7.48 t	7.49* t	7.48 m
3	7.27 t	7.32 t	7.30 t	7.27 t	7.28 t	7.28 t
4	9.30 d	9.31 d	9.32 d	9.31 d	9.33 d	9.29 d
6	8.53 s	8.62 s	8.16 s	7.85 s	8.27 s	8.57 s
7	4.96 s	4.98 s	4.98 s	4.97 s	5.00 br s	4.99 s
8	7.96 d	7.93 d	7.98 d	7.93 d	8.04 d	8.05 d
9	7.27 t	7.35 t	7.36 t	7.34 t	7.34 t	7.34 t
10	7.41 t	7.48 t	7.48 t	7.45 t	7.47* t	7.48 m
11	7.98 d	8.02 d	8.05 d	8.02 d	8.04 d	8.05 d
3′	4.04 d	4.68 d	4.36 dd	4.26 br s	4.28 d	4.21 d
4′	3.24 m	4.53 ddd	4.57 br d	3.13 m	3.41 m	3.08 ddd
5′a	2.49 m	3.13 ddd	2.85 dddd	2.48 m	2.88 m	2.89 m
5′b	2.49 m	2.98 dd	2.51 m	2.40 m	2.47 m	2.21 m
6′	6.68 s	7.07 br d	6.97 dd	6.92 dd	6.82 dd	6.82 dd
2′-CH_3	2.29 s	2.44 s	2.41 s	2.43 s	2.42 s	2.42 s
3′-OCH_3	3.32 s	3.60 s	2.77 s	2.80 s	2.98 s	2.69 s
4′-NCH_3	1.44 s			2.65 s	2.27* s	2.38 s
1″			8.12 s	8.18 s		
2″a					4.55 d	
2″b					4.46 d	
5″					1.76* s	
N-OH			9.39 br s			7.78 br s
Some characteristic coupling-constants (Hz):						
1, 2	8	8	8	8	8	
3, 4	8	8	8	8	8	
8, 9	7	8	8	8	8	
10, 11	8		8	8	8	
3′, 4′	3	10.5	3		4	
4′, 5′a/b		13/3	12/5			
5′a, 5′b		13	13	14		
5′a/b, 6′		5/1	6/9	6/9		
2″a, 2″b					8	

Chemical shifts given in ppm. Solvent DMSO-d_6. Assignments with asterisks may be interchanged. Recorded at [a] 30°C, [b] 100°C, [c] 150°C, [d] 80°C. [e] Ref 10), [f] Hydrolysis product of **6**.

^{13}C NMR spectra are compiled in Table 2. The numbering system is the same as used in Ref.[6].

4′-Demethylamino-4′-hydroximinostaurosporine, TAN-1030A (**1**)

The pooled eluate fractions containing pure by-product **1** were recrystallized from ethyl acetate-methanol. The almost colorless crystals melted under decomposition at 238~44°C. NMR and MS data were virtually identical with the published ones for TAN-1030A[10]. In order to corroborate the stereochemistry of **1** the 6,*O*-dimethyl derivative (**9**) was prepared by stirring oxime **1** with an excess of methyl iodide and silver oxide for 2 days. NOE experiments (Fig. 2) revealed that the 2′-Me group and the 3′-H must be located on the same side of the pyrane ring system. The syn configuration of the oxime functionality relative to the methoxy group as deduced in Ref.[13] could be confirmed.

4′-Demethylamino-4′-nitro-staurosporine (**2**)

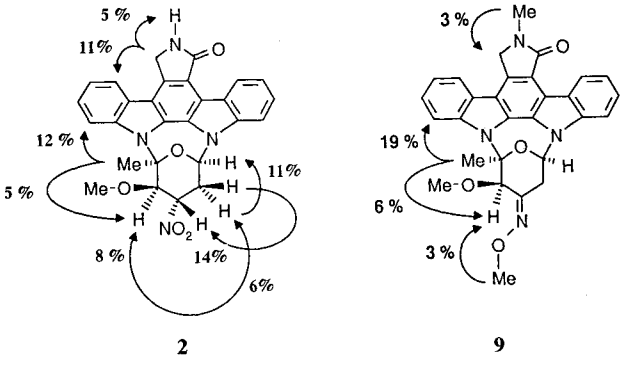
The ^{1}H NMR spectra of staurosporine and its by-product **2** are rather similar. The signal of the N–CH_3 group is missing in the spectrum of **2** and that of H-4′ is strongly upshifted from 3.2 to 4.5 ppm. Comparison of the elementary composition shows the presence of 2 additional oxygen atoms. In conjunction with the ^{1}H NMR data this finding suggests the presence of a nitro group in position 4′. This conclusion was supported by a characteristic, very strong absorption band in the IR spectrum at 1560 cm^{-1}. The proposed structure of **2** is further supported by the ^{13}C NMR data in Table 2 which show the expected shifts for the signals of carbons 3′, 4′ and 5′. The analysis of the coupling constants suggests a chair conformation of the sugar moiety and an equatorial position of the nitro group. The coupling constant of 11 Hz between H-3′ and H-4′ is much larger than in other staurosporine derivatives (4 Hz) and is fully

Table 2. ^{13}C NMR chemical shifts of compounds **1**, **2**, **3**, **4** and **6**.

Carbon	1[e]	2[a]	3[b]	4[c]	6[d]
1	108.2 d	109.8 d	108.7 d	108.8 d	108.6 d
2	124.8 d	126.1 d	125.2 d	125.2 d	125.0 d
3	118.9 d	120.4 d	119.4 d	119.3 d	119.2 d
4	125.5 d	126.2 d	125.8 d	125.6 d	125.8 d
4a	122.4 s	123.4 s	123.0 s	122.6 s	122.8 s
4b	114.0 s	115.8 s	115.4 s	115.1 s	114.7 s
4c	118.7 s	120.2 s	119.5 s	≈119.3	119.2 s
5	172.2 s	172.2 s	171.9 s	171.8 s	172.0 s
7	45.3 t	45.9 t	45.4 t	45.3 t	45.4 t
7a	131.9 s	133.1 s	132.6 s	132.5 s	133.3 s
7b	113.4 s	115.1 s	114.2 s	114.0 s	114.0 s
7c	123.8 s	124.5 s	124.1 s	123.7 s	124.0 s
8	120.7 d	121.7 d	121.3 d	121.4 d	121.1 d
9	119.6 d	121.1 d	120.3 d	120.2 d	120.1 d
10	124.2 d	125.7 d	124.9 d	124.9 d	124.7 d
11	115.1 d	115.7 d	113.3 d	113.1 d	113.9 d
11a	139.3 s	140.6 s	138.8 s	138.5 s	138.7 s
12a	129.9 s	127.9 s	129.7 s	129.2 s	130.0 s
12b	126.6 s	125.2 s	125.6 s	125.2 s	126.3 s
13a	136.3 s	136.9 s	136.5 s	136.2 s	136.3 s
2′	91.0 s	95.3 s	94.2 s	94.5 s	92.9 s
3′	82.7 d	85.4 d	82.0* d	83.4 d	82.8 d
4′	50.0 d	80.6 d	53.6 br	52.9 b	60.0 d
5′	29.3 t	35.2 t	26.9 t	27.6 t	29.4 t
6′	79.8 d	81.1 d	81.8* d	82.0 d	80.8 d
2′-CH_3	29.6 q	28.8 q	29.1 q	28.6* q	28.4 q
3′-OCH_3	57.1 q	62.4 q	60.2 q	60.0 q	58.3 q
4′-NCH_3	33.2 q			28.1 q	44.4 q
1″			159.7 br s	162.8 s	
2″					89.8 t
4″					169.0 s
5″					20.3 q

Chemical shifts given in ppm. Solvent DMSO-d_6. Assignments with asterisks may be interchanged. Recorded at [a] 30°C, [b] 100°C, [d] 80°C, [c] recorded at 40°C, major signals. The minor signals were within ±0.5 ppm except 4′: 46.6 ppm; 5′: 26.1 ppm; 2′-Me: 31.4* ppm; 4′-NCH_3: 28.9* ppm; 1″: 163.3 ppm. [e] Ref 10).

Fig. 2. Characteristic NOEs of 4′-demethyl-4′-nitro-staurosporine (**2**) and the dimethyl TAN 1030A derivative (**9**).



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compatible with an anti relationship. The axial position of the protons 3′ and 5′a is corroborated by NOE experiments showing 8% and 6% enhancement (see Fig. 2). The nitro staurosporine analogue **2** constitutes another representative of the rather small group of secondary metabolites containing an aliphatic nitro group[14].

4′-Demethyl-*N*-formyl-*N*-hydroxy-staurosporine (**3**)

The HRFAB-MS (listed in the section Physico-chemical Data) is in accordance with the molecular formula $C_{28}H_{24}N_4O_5$. The ^1H NMR spectrum reveals an intact aglycone moiety of staurosporine and the lack of the N–CH_3 group. In addition two rather broad signals are observed at 9.4 and 8.1 ppm. The assignment of the latter signal to an *N*-formyl group is confirmed by the presence of a broad ^{13}C NMR signal at 160 ppm. The presence of a formyl ester can be excluded, since no ester carbonyl absorption above 1700 cm^{-1} is observed in the IR spectrum. A very broad IR-absorption band at 3400 cm^{-1} indicates the presence of a hydroxy group. This group can only be linked to the 4′-nitrogen atom as part of the hydroxamic acid function in the structural formula **3**.

N-Formyl-staurosporine (**4**)

The molecular formula $C_{29}H_{26}N_4O_4$ indicates the presence of an addition carbonyl in comparison to staurosporine. The ^{13}C NMR signal at 162.8 ppm is again in accordance with the presence of an *N*-formyl group. The position of this formyl group is clarified by analysis of the ^1H NMR spectrum: The signal of the 4'-*N*-methyl group recorded at 30°C is split into two separate signals due to the syn and anti conformations of the formyl group. At 150°C only a single signal is observed as a result of a rapid conformational interchange.

Staurosporine By-product **6**

The analysis of the ^1H and ^{13}C NMR spectra suggests again the presence of a staurosporine substructure with an additional substituent at the 4'-amino group. Its elementary composition differs by an additional $C_3H_4O_3$ residue of which all protons can clearly be seen in the ^1H NMR as a singlet methyl group at 1.76 ppm and a doublet at 4.55 and 4.46 ppm of a methylene group. The former is part of a terminal acetate function characterized by two ^{13}C NMR signals at 20.3 and 169.0 ppm. The position of the CH_2O link could be established by hydrolysis of **6** with Na_2CO_3 in methanol at room temperature. The FAB-MS of the resulting product **10** was fully compatible with that predicted for *N*-hydroxy-staurosporine and the ^1H NMR signals for the methyl and methylene group mentioned above had disappeared (Table 1).

Biological Acitivity

The new staurosporine derivatives **2**, **3**, **4** and **6** were tested against various protein kinases (see Table 3). Porcine PKC[8] was inhibited in the same range as by the known metabolite K-252a[15]. These IC_{50}-values are considerably higher than the IC_{50}-value of 6 nM obtained with staurosporine. In addition, the selectivity pattern against different kinases is rather undesirable: The new compounds are several times more active against phosphorylase kinase (4 ~ 200 nM) than against the target enzyme PKC.

With respect to the biosynthetic pathway for the formation of the 4'-nitro derivative **2** the by-products identified in the course of this work provide rather limited information. The oxime TAN-1030A (**1**) appears to be a key intermediate which could be a precursor as well as a degradation product of staurosporine. This question will be addressed in a forthcoming publication[16]. The nitro group of **2** could be formed by further oxidation of the oxime **1** or *via* the reduced hydroxylamino derivative **8**. The latter is of limited stability in the fermentation broth and can be isolated only in its acylated form **3**. An analogous formylation reaction of staurosporine itself leads to the formyl derivative **4**, the partial synthesis of which from staurosporine has already been described in the patent literature[11].

Table 3. Enzyme inhibition of proteine kinases.

Name	IC_{50} (μM)		
	PKC	PKA	PK
Staurosporine (**5**)	0.006	0.015	0.003
TAN-1030$_A$ (**1**)	1.2	1.4	0.04
4'-Demethylamino-4'-nitro-staurosporine (**2**)	0.35	50	0.07
4'-Demethyl-*N*-formyl-*N*-hydroxy-staurosporine (**3**)	0.1	0.1	0.004
N-Formyl-staurosporine (**4**)	0.09	0.5	0.02
Compound **6**	0.7	1.1	0.2
CGP 41'251 *N*-benzoyl-staurosporine	0.05	2.4	0.048

PKC: Protein kinase C.
PKA: Cyclic AMP-dependent protein kinase.
PK: Phosphorylase kinase.

In contrast to all other known substituents in position 4' the nitro group of **2** assumes an equatorial position. The epimerization reaction to this more stable confirmation could already occur during the fermentation or during the isolation steps as a result of the acidity of the proton in position 4'. In preliminary derivatization experiments, the nitro group of **2** could not be reduced to the hydroxylamine (**8**) or the corresponding amine. With all reagents examined, the oxime **1** was the only product which could be identified in the reaction mixtures[16].

Further exploration of the biosynthetic pathway for the formation of staurosporine will clearly require isolation and systematic investigations of blocked mutants of the producer strain *Streptomyces longisporoflavus* R19. A manuscript describing the final biosynthetic step is already in preparation[17].

Physico-chemical Data

Data of **2**: White powder from dichloromethane/isopropanol, MP 237 ~ 242°C HREI-MS: *m/z* 482.1595 ($C_{27}H_{22}N_4O_5$, δm 0.5 mmu) EI-MS: *m/z* 482 (8, M^+), 450 (5), 436 (4), 394 (15), 362 (20), 348 (19), 311 (50), 310 (24), 283 (20), 282 (30), 281 (18), 255 (18), 140 (42), 125 (26), 122 (35), 110 (19), 97 (20), 83 (53), 69 (30), 43 (100), IR (KBr) cm^{-1} 3400, 1680, 1560, 1450, 1390, 1370, 1350, 1310, 1270, 1230, 1160, 1120, 1050, 1020, 960, 830, 760, 740, 660 CD (λ^{EtOH} nm (θ): 364 (1,500), 336 (1,500),

295 (16,000), 263 (0), 252 (14,000), 230 (7,800), 209 (−23,000).

Data of **3**: White powder lyophilyzed from acetonitrile/water, MP 220°C (dec.) HRFAB-MS: *m/z* 497.1822 ($M+H^+$, $C_{28}H_{25}N_4O_5$, δm 0.3 mmu) IR (KBr) cm^{-1}: 3400, 2920, 2860, 1665, 1580, 1480, 1460, 1420, 1395, 1370, 1345, 1320, 1285, 1270, 1250, 1225, 1180, 1150, 1140, 1120, 1100, 1070, 1050, 1040, 775, 745, 610.

Data of **4**: Colorless crystals from ethyl acetate/dichloromethane, MP 221~6°C HREI-MS: *m/z* 494.1951 ($C_{29}H_{26}N_4O_4$, δm 0.3 mmu) EI-MS: *m/z* 494 (33), 466 (4), 435 (10), 379 (22), 349 (82), 348 (100), 337 (58), 311 (19), 310 (16), 282 (20), 281 (19), 125 (36), 43 (56) IR (KBr) cm^{-1} 3420, 2920, 2840, 1670, 1460, 1400, 1340, 1310, 1280, 1250, 1230, 1150, 1120, 1070, 745

Data of **6**: Colorless crystals from dichloromethane/isopropanol, MP 140~3°C HREI-MS: *m/z* 554.2153 ($C_{31}H_{30}N_4O_6$, δm 1.2 mmu) EI-MS: *m/z* 554 (2), 466 (6), 349 (10), 348 (12), 337 (10), 327 (7), 311 (9), 234 (17), 219 (17), 91 (18), 60 (35), 45 (57), 43 (100) IR (KBr) cm^{-1}: 3410, 3040, 2920, 2860, 1740, 1680, 1630, 1585, 1455, 1400, 1370, 1350, 1320, 1280, 1230, 1150, 1120, 1100, 1015, 955, 745. CD (λ^{EtOH} nm (θ): 372 (−790), 366 (1,400), 348 (740), 296 (20,000), 264 (−5,300), 252 (10,500), 242 (0), 233 (6,600), 213 (−38,000).

Data of **10**: FAB-MS: *m/z* 483 ($M+H^+$).

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