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The Coumarin-Binding Site in Carbonic Anhydrase Accommodates Structurally Diverse Inhibitors: The Antiepileptic Lacosamide As an Example and Lead Molecule for Novel Classes of Carbonic Anhydrase Inhibitors†

Claudia Temperini,‡ Alessio Innocenti,‡ Andrea Scozzafava,‡ Seppo Parkkila,§,∥ and Claudiu T. Supuran*,‡

‡Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy, §Institute of Medical Technology, and ∥School of Medicine, University of Tampere and Tampere University Hospital, Tampere, Finland

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Coumarins constitute a general and totally new class of inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1), binding at the entrance of the active site cavity. We report here that the coumarin-binding site in CAs may interact with diverse compounds, such as the antiepileptic drug lacosamide, which inhibits mammalian CAs I−XV, with inhibition constants in range of 331 nM to 4.56 μM. Its X-ray crystal structure in adduct with CA II reveals the molecular basis for this inhibition. Lacosamide was found in the coumarin-binding site, making favorable van der Waals interactions with Thr200, Asn67, Gln92, and Phe131. No interactions with the Zn(II) ion were evidenced in the CA II−lacosamide adduct. The coumarin-binding site may thus accommodate structurally diverse compounds which possess an inhibition mechanism distinct of that of sulfonamides. This finding opens new possibilities for designing CA inhibitors/activators with various biomedical applications.

Introduction

Carbonic anhydrases (CAs,[a] EC 4.2.1.1) are thoroughly investigated zinc enzymes due to their involvement in many physiologic and pathologic processes.[1,2] Indeed, the 16 different isoforms described so far in mammals, including *Homo sapiens*, are involved in pH and CO_2 homeostasis, respiration, and transport of CO_2/bicarbonate between metabolizing tissues and lungs, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (e.g., gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other processes.[1−5] The Zn(II) ion of CAs is critical for the catalytic cycle of these enzymes, being coordinated by three His residues and a water molecule/hydroxide ion, which acts as a nucleophile in the conversion of carbon dioxide to bicarbonate and a proton, the physiological reaction catalyzed by these enzymes.[1−3] Inhibition of CAs is used in clinical medicine for more than 50 years,[2] with the main class of inhibitors being constituted by sulfonamides and their bioisosteres, such as the sulfamates, sulfamides, etc.[1−5] Sulfonamides and their isosteres bind to the Zn(II) ion within the CA active site, displacing the water molecule/hydroxide ion.[1−3] At least 30 clinically used drugs show significant inhibition of all 16 mammalian CA isoforms but not isoform-selective inhibitors are known to date.[2] CA inhibitors (CAIs) are used as diuretics, antiglaucoma, anticonvulsant, antiobesity, and antitumor drugs/diagnostic agents, and different isoforms are targeted for such diverse applications.[1−3] Some of these compounds used as drugs such as sulfanilamide **1**, acetazolamide **2**, zonisamide **3**, and topiramate **4** are shown below. However, the lack of selectivity for the target isozyme constitutes a main problem with the sulfonamide/sulfamate/sulfamide CAIs,[2] and this led to the search of different chemotypes, which may show potent enzyme inhibitory activity and a more selective inhibition profile. Indeed, we recently reported a novel class of CAIs belonging to a completely new chemotype, the coumarins, and deciphered the inhibition mechanism of these inhibitors.[6] The natural product[6a,7] coumarin 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2*H*-chromen-2-one **5**, as well as structurally related, simple coumarin derivatives possessing various substitution patterns at the heterocyclic ring,[6b] were shown to be hydrolyzed within the CA active site with formation of 2-hydroxy-cinnamic acids (**6** is formed from the natural product **5**), which possess significant CA inhibitory properties and bind in a completely unprecedented manner to the enzyme, not interacting with the Zn(II) ion as the sulfonamide/sulfamate/sulfamide type of inhibitors (Scheme 1).[6]

The new inhibitor-binding site evidenced in the preceding work,[6] i.e., the coumarin-binding site, might, in principle, accommodate other inhibitors, affording thus an opportunity for drug design campaigns of novel CAIs. Indeed, for example, we have recently shown that the protein tyrosine kinase inhibitors with antitumor activity (and clinically used drugs) imatinib and nilotinib are low nanomolar CA I and II inhibitors.[8] However, we were unable to understand the detailed inhibition mechanism with these two drugs, as no X-ray crystal structure of imatinib/ nilotinib in adduct with CA could be obtained. Here we investigate the interaction of the new antiepileptic lacosamide **7**, (2*R*)-2-acetylamino-*N*-benzyl-3-methoxypropanamide, discovered by Kohn's group and possessing a distinct mechanism of action compared to any

†The X-ray coordinates of the hCA II−lacosamide adduct are available in PDB with the ID code 3IEO.

*To whom correspondence should be addressed. Phone: +39-055-457 3005. Fax: +39-055-4573385. E-mail: claudiu.supuran@unifi.it.

[a]Abbreviations: CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; hCA, human CA; mCA, murine CA.

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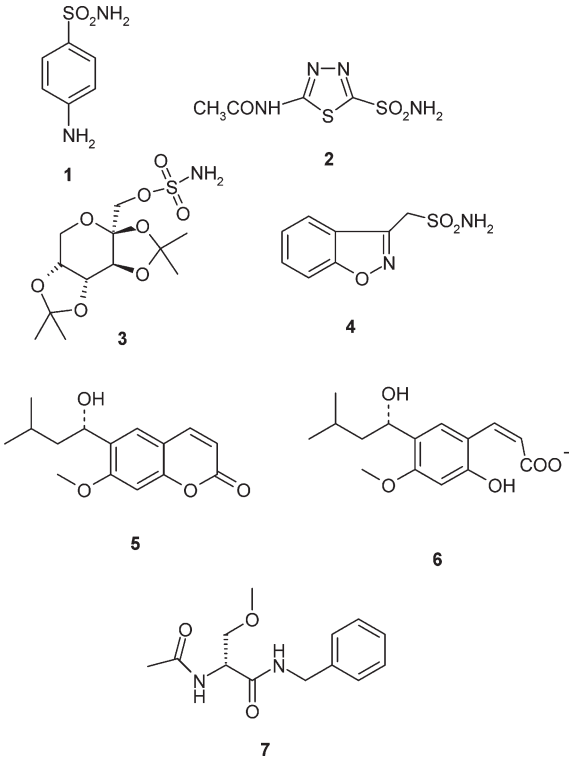
Scheme 1. Structures **1**−**7**



other antiepileptic drug,[9,10] with CAs. Although lacosamide does not possess any moiety typically found in CAIs investigated so far, such as the sulfonamide, sulfamate, sulfamide or coumarin ones, we show here that it acts as an effective inhibitor of all mammalian CA isozymes CA I−XV and binds to isoform CA II active site similarly to the hydrolyzed coumarins,[6] not interacting with the metal ion. These findings may have relevance both for understanding the mechanism of action of this new pharmacological agent, as well as for the design of CAIs, which do not interact directly with the metal ion from the enzyme active site. In fact, all clinically used CAIs known to date do indeed bind to the Zn(II) ion from the enzyme cavity, as mentioned above.

Results and Discussion

Chemistry and CA Inhibition. The rationale for investigating lacosamide **7** as a potential CAI is based on the following three assumptions:

(i) Several antiepileptics, such as topiramate **3** and zonisamide **4** show potent CA inhibitory activity. Obviously, these compounds possess the sulfamate or sulfonamide groups which bind directly to the Zn(II) ion within the CA active site, as shown by X-ray crystallography and detailed kinetic measurements for both these drugs, against all mammalian CA isozymes (see also Table 1);[11]

(ii) Although the shape of the lacosamide molecule, and that of the coumarin hydrolysis product of the 2-hydroxycinamic acid type are not very similar, they may be considered to have something in common. Thus, both **6** and **7** possess at least an aromatic ring and an elongated side chain moiety of the hydroxypentyl type in **6** and slightly longer, incorporating 7 atoms, in lacosamide **7**. However, there are also important difference between **6** and **7**, as the first compound incorporates two long arms and two smaller substituents at the phenyl ring (OH and OMe groups), whereas the last one has only one, longer arm substituting the phenyl ring and no smaller polar groups such as OH, OMe, etc.

(iii) Lacosamide **7** seems to modulate the slow inactivation gate sodium channels[12a,b] and collapsin response mediator protein 2[12c] as antiepileptic mechanisms, but high affinity binding targets for this drug have not been identified so far, even if 100 potential binding sites in various receptors have been investigated.[12d] Thus, the cognate receptors/proteins with which the drug may interact in vivo are largely unknown, although a lot of interesting research was recently reported by its discoverers, Kohn's group.[12d] We thus hypothesized that CAs might be a putative target of this new drug.

Table 1. Inhibition of CA Isozymes I−XV with Compounds **1**−**7**, by a Stopped-Flow CO_2 Hydrase Assay at 25 °C[13]

	K_I (nM)[b]					
isoform[a]	**1**[c]	**2**[c]	**3**[d]	**4**[e]	**5**[f]	**7**[g]
hCA I	25000	250	250	56	78	362
hCA II	240	12	10	35	59	331
hCA III	988	2×10^5	7.8×10^5	2.2×10^6	>500000	374
hCA IV	9760	74	4900	8590	3800	525
hCA VA	32000	63	63	20	96000	4565
hCA VB	3650	54	30	6033	17700	341
hCA VI	941	11	45	89	35700	412
hCA VII	70	2.5	0.9	117	27900	4446
hCA IX	238	25	58	5.1	54500	353
hCA XII	37	5.7	3.8	11000	48600	3713
hCA XIII	32	17	47	430	7860	1210
hCA XIV	5400	41	1460	5250	7800	473
mCA XV	9610	72	78	634	93100	461

[a] h = human, m = murine isoform; all proteins were recombinant ones obtaine in-house. [b] Errors in the range of ±5% of the reported data from three different assays. [c] From ref 16. [d] From ref 11a. [e] From ref 11b. [f] From ref 6. [g] This work.

To test our working hypothesis, we assayed the in vitro inhibition[13] of mammalian isoforms CA I−XV with lacosamide **7** and compared these data with those of the sulfonamides **1**, **2**, and **4**, the sulfamate **3**, and the coumarin **5** (hydrolyzed to the *cis*-2-hydroxycinnamic acid **6**)[6a] (Table 1).[11,14−16]

It may be observed from data of Table 1 that lacosamide **7** is a medium potency inhibitor of isoforms hCA I, II, III, IV, VB, VI, IX, XIV, and mCA XV, with inhibition constants in the range of 331−525 nM. The remaining isozymes, i.e., hCA VA, VII, XII, and XIII, are less inhibited by this drug, with inhibition constants in the micromolar range (K_Is of 1.21−4.56 μM). By comparing lacosamide **7** with the coumarin **5** as CAIs, it may be observed that the coumarin is a more effective inhibitor of CA I and II (K_Is of 59−78 nM), and much less effective against all other isoforms, compared to lacosamide **7**. Lacosamide is the best hCA III inhibitor among all compounds investigated here, such as the sulfonamides **1**, **2**, and **4** or the sulfamate **3** and coumarin **5**, whereas the sulfonamides/sulfamates generally act as more effective CAIs against other isoforms than CA III, compared to lacosamide. Indeed, for example, the heterocyclic sulfonamide acetazolamide **2** has inhibition constants <75 nM against all isoforms except CA I and III. The aromatic sulfonamide sulfanilamide **1** on the other hand is a much

weaker CAI, effectively inhibiting (K_Is < 70 nM) only CA VII, XII, and XIII (Table 1). All these data show unambiguously that lacosamide, unexpectedly, is a CAI with an efficacy intermediate between that of heterocyclic sulfonamides/sulfamates (**2** and **3**) and aromatic ones (**1**) against many CA isoforms. Furthermore, lacosamide is a more efficient CAI compared to coumarin **5** against all isoforms except CA I and II.

Inhibition Mechanism and X-ray Crystallography. To understand the inhibitory mechanism of lacosamide, a compound not possessing moieties generally present in CAIs, we resolved the X-ray crystal structure (at a resolution of 2.0 Å) of the drug **7** in adduct with the physiologically dominant CA isoform, hCA II.[2,14]

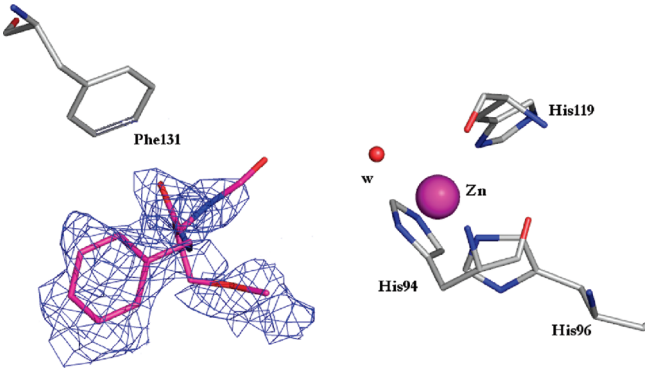


Figure 1. Omit map of lacosamide **7** (magenta and blue) bound within the hCA II active site. The Zn(II) ion (violet sphere), its three coordinated histidine residues (His94, 96, and 119), and water molecule (red sphere, labeled w) as well as the Phe131 side chain are also shown.

Inspection of the electron density maps (Figure 1) at various stages of the refinement showed features compatible with the presence of one molecule of inhibitor bound within the active site (Figure 1), and as for the previously reported structure of coumarin **5** in adduct with hCA II,[6] the inhibitor does not interact with the metal ion from the bottom of the enzyme active site but is accommodated at its entrance (Figures 1 and 2). All atoms of lacosamide **7** have clearly defined electron density in the hCA II−**7** adduct (Figure 1). The crystallographic parameters and refinement statistics of the adduct are presented in Table 2.

Lacosamide **7** adopts an extended conformation when bound to the hCA II active site, making no hydrogen bonds at all but only hydrophobic van der Waals interactions (<4 Å) with several amino acid residues, such as Thr200, Phe131, Gln92, and Asn67, known to interact with other classes of inhibitors (sulfonamides, sulfamates, coumarins), from previous X-ray crystallographic work.[6,14,15] Indeed, the acetamido moiety of lacosamide is orientated toward Gln92, with the distance between the methyl group of the inhibitor and the NH_2 moiety of Gln92 being of only 2.2 Å. The methyl belonging to the methoxy moiety of **7** is, on the other hand, orientated toward Asn67, with a distance between the oxygen of the $CONH_2$ moiety of Asn67 and the methyl of the inhibitor of 3.7 Å. Also the $PhCH_2$ fragment of lacosamide interacts with this amino acid residue, i.e., Asn67, with a distance of 4.0 Å between the same carbonyl oxygen of the amino acid residue and the methylene moiety of **7**. On the other hand, many atoms of the phenyl ring of Phe131 are in van der Waals contacts with the $CONH-CH_2$ and phenyl fragments of lacosamide (data not shown). Thr200 (the OH moiety) is also in van der Waals contact with the methoxy moiety of lacosamide, whereas the phenyl

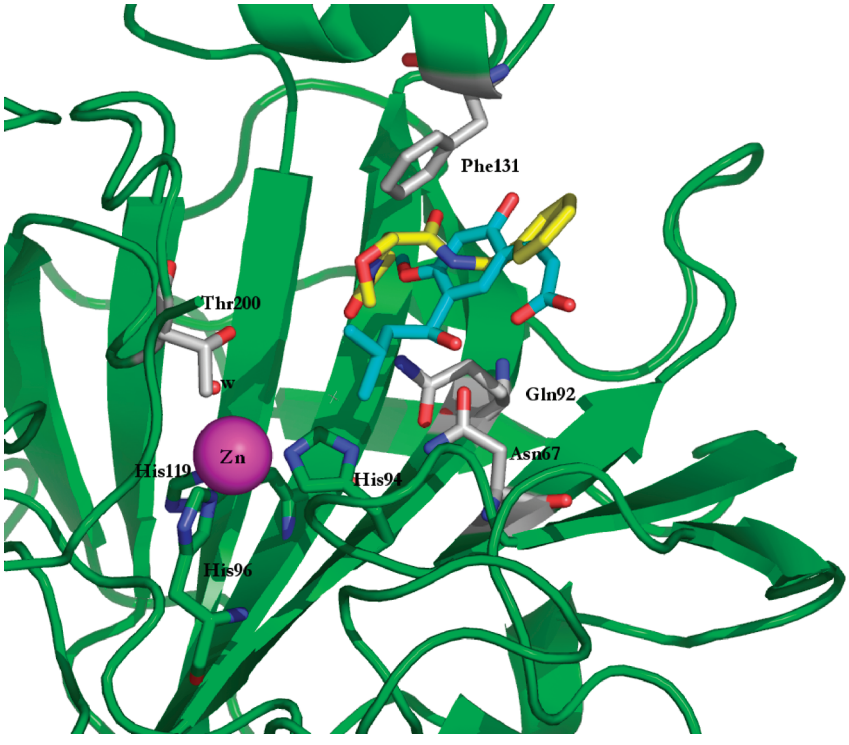


Figure 2. Superposition of the hCA II−hydrolyzed coumarin **6** (in blue) adduct (PDB file 3F8E)[6a] with the hCA II−lacosamide (yellow) adduct (PDB file 3IEO, this work). The Zn(II) ion is the central violet sphere with its three coordinated histidine residues (His94, 96, 119) shown, whereas the protein backbone is represented as green ribbon. Amino acid residues involved in the binding of inhibitors **6** and **7** are shown in detail (Thr200, Asn67, Gln92, and Phe131, CPK colors).

Table 2. Crystallographic Parameters and Refinement Statistics for the hCA II−Lacosamide **7** Adduct

parameter	value
Crystal Parameter	
space group	$P2_1$
cell parameters	a = 42.19 Å
	b = 41.55 Å
	c = 72.23 Å
	β = 104.5°
Data Collection Statistics (20.0−2.0 Å)	
no. of total reflections	27444
no. of unique reflections	16696
completeness (%)[a]	99.0 (99.8)
$F2/\mathrm{sig}(F2)$	21.0(4.7)
R_{sym} (%)	10.0 (22.0)
Refinement Statistics (20.0−2.0 Å)	
R factor (%)	25
R_{free} (%)[b]	29
rmsd of bonds from ideality (Å)	0.018
rmsd of angles from ideality (deg)	1.8

[a] Values in parentheses relate to the highest resolution shell (2.1−2.0 Å). [b] Calculated using 5% of data.

moiety of **7** is orientated toward the exit of the active site cavity, not making many interactions with other amino acids than Phe131 (from which it is anyhow at around 4−4.5 Å). Thus, this moiety of **7** can surely be derivatized in order to augment the affinity of the inhibitor for the CA active site. Indeed, by introducing diverse moieties as substituents at this phenyl ring, there would be enough space for a new inhibitor to bind effectively to CA and make more interactions with amino acid residues on the rim of the entrance to the active site. From the superposition of the hCA II−coumarin **5** and hCA II−lacosamide **7** adducts (Figure 2), it may be observed that even if the active site region where the two compounds bind is the same, the two adducts are not entirely superposable. The stronger inhibitor **5** makes many more polar interactions with various amino acid residues and water molecules from the hCA II active site,[6] presumably because the four polar side chains substituting its phenyl ring (obtained after the hydrolysis of the coumarin **5** to the corresponding *cis*-2-hydroxycinnamic acid derivative **6**) allow the latter compound to participate in many other interactions compared to the unique side chain present in lacosamide **7**. This may explain why coumarin **5** is 5.6 times a better hCA II inhibitor compared to lacosamide **7**. However, the bulky moieties of **5** probably interact in an unfavorable way with amino acid residues from other isoforms than CA I and II, explaining thus its weaker inhibitory activity against the other isoforms[6] (Table 1). Thus, by introducing a different number of side chains possessing a diverse chemical nature at the phenyl ring of lacosamide **7**, it might be possible to modulate extensively both the activity and isoform selectivity of compounds which bind within the coumarin-binding site of the CAs. This opens new vistas for the design of different types of CAIs based on this very interesting lead, lacosamide **7**.

Conclusions

We report here a detailed investigation of all mammalian CA isoforms inhibition with lacosamide, an antiepileptic drug recently approved (in the fall of 2008) for the treatment of partial-onset seizure and diabetic neuropathic pain (marketed with the name Vimpat), possessing a mechanism of antiepileptic action distinct from all other such drugs and poorly understood at this moment. Here we prove that lacosamide inhibits all mammalian CA isozymes I−XV, with inhibition constants in range of 331 nM to 4.56 μM. Its X-ray crystal structure in adduct with the physiologically dominant isoform CA II revealed the molecular basis for this inhibition. Lacosamide was found in the coumarin-binding site, at the entrance of the CA II active site, making favorable interactions with Thr200, Asn67, Gln92, and Phe131. No interactions with the Zn(II) ion were evidenced in the CA II−lacosamide adduct, as for the coumarin CAIs. The coumarin-binding site may thus accommodate structurally diverse compounds which possess an inhibition mechanism distinct of that of sulfonamides, sulfamates, or similar zinc-binders. This finding opens new possibilities for designing CA inhibitors/activators with various biomedical applications.

Experimental Protocols

Chemistry. Lacosamide (Vimpat) was from UCB (Brussels, Belgium). Compounds **1**−**5** are commercially available (Sigma-Aldrich, Milan, Italy) or were reported earlier by our group.[6] CA isozymes were recombinant ones prepared in our laboratory as reported earlier.[16]

CA Inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[13] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5−10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled−deionized water and dilutions up to 0.01 nM were done thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min to 6 h at room temperature (15 min) or 4 °C (all other incubation times) prior to assay in order to allow for the formation of the E−I complex or for the eventual active site-mediated hydrolysis of the inhibitor. Data reported in Table 1 show the inhibition after 15 min incubation (except for coumarin **5**, for which an incubation of 6 h has been used).[6] Longer incubation periods (than 15 min) between lacosamide **7** and CAs did not change the K_Is values of this compound (data not shown). The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, as reported earlier,[1,6,7] and represent the mean from at least three different determinations.

Crystallization, X-ray Data Collection, and Refinement. Crystals of the hCA II−**7** complex were obtained by using the hanging-drop method for cocrystallizing the protein with the ligand, as previously described.[6] A monochromatic experiment at the Cuα wavelength was performed on a crystal of hCA II grown in the presence of lacosamide by the rotation method on a PX-Ultra sealed-tube diffractometer (Oxford Diffraction) at 100 K. The crystal belonged to space group $P2_1$ (a = 42.19; b = 41.550; c = 72.23 β = 104.56). Data were processed with CrysAlis RED (Oxford Diffraction).[17] The structure was analyzed by difference Fourier technique, using the PDB file 1CA2[4b] as the starting model. The refinement was carried out with the program REFMAC5,[18] and model building and map inspections were performing using the COOT program.[19] The final model of the hCA II−lacosamide **7** complex had an R factor of 25% and R_{free} 29.0% in the resolution range

20.0−2.0 Å, with a rms deviation from standard geometry of 0.018 Å in bond lengths and 1.8° in angles. The correctness of stereochemistry was checked using the program PROCHECK.[20] The coordinates of the hCA II−lacosamide adduct are available in PDB, ID code 3IEO.

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